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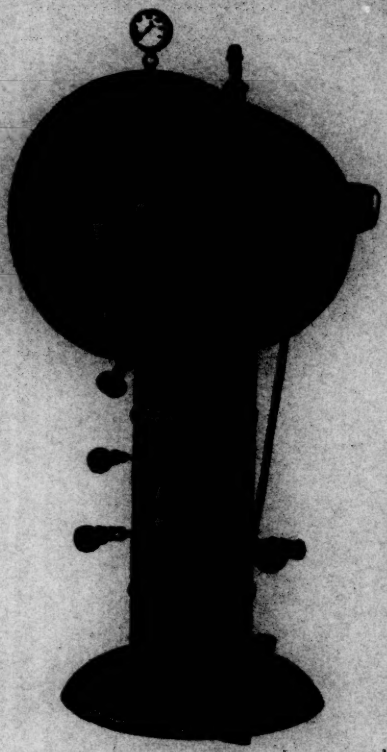

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THE RATE OF DIALYSIS OF THE BLOOD SUGAR IN EXPERIMENTAL DIABETES.*

By ISRAEL S. KLEINER.

(From the Department of Physiology and Pharmacology of The Rockefeller Institute for Medical Research.)

(Received for publication, April 30, 1918.)

Dialysis methods have been used by many investigators in the attempt to determine whether the sugar in the blood is "free" or "combined;" that is, whether it is a crystalloid in simple solution or exists in some more or less stable union with another substance. If it is free it will pass through a membrane permeable to sugar but if it is combined, the colloidal molecule presumably formed should be held back. The technique of these various investigations has differed in many particulars,—in the proportion of the volume of the blood to that of the saline solution, in the manner of preventing clotting, in the composition of the saline solution, in the duration of dialysis, temperature, type of membrane, etc. In two respects, however, practically all resemble each other.

First, all have used the blood of normal animals exclusively. This is probably because of the conception that since normal blood sugar does not pass the kidneys it is in a non-diffusible state. However, the property of being secreted by the kidney is not necessarily a criterion of diffusibility. Various colloidal substances when administered parenterally are quickly thrown out by the kidney; the higher polysaccharides[1] are good examples. On the other hand, one frequently observes hyperglycemia without glycosuria both in human and in experimental diabetes, which would indicate, according to the above hypothesis, the occurrence

* A preliminary report of this work was presented before the Society for Experimental Biology and Medicine, March 20, 1918.

[1] Mendel, L. B., and Mitchell, P. H., *Am. J. Physiol.*, 1905, xiv, 239.

of combined sugar in some diabetic bloods. Therefore, it seems evident that if combined sugar occurs there is as great a possibility of finding it in diabetic as in normal blood. The higher percentage of sugar in diabetic blood is an advantage from an analytical standpoint which will be readily appreciated.

Secondly, all previous workers have determined the sugar content only at the beginning and at the end of the dialyzing period, which has usually been either 24 or 48 hours. Most of them[2] have found that normal blood sugar is completely, or nearly completely, removed by a sufficiently long period of dialysis. Lépine and Boulud[3] found no sugar dialysis in 2 hours under suitable conditions but lately Lépine[4] has stated that certain blood samples may contain both "free" and "combined" sugar, both of which can be shown to dialyze; it must be noted however that Lépine's "combined" sugar is that which is split off by heating with tartaric acid. Consequently although it cannot be said that the question is settled, the preponderance of opinion is that the sugar dialyzes freely under these conditions.

Michaelis and Rona[5] have pointed out, however, that if both free and combined sugar exist side by side in the blood they are probably in equilibrium with one another. When the blood is dialyzed the free sugar will pass out, thus destroying the equilibrium and forcing the decomposition of a part of the combined sugar. The new free sugar formed will now dialyze and thus eventually as much sugar will be lost as if all the sugar originally present had been free. Therefore the complete disappearance of sugar from a dialyzing specimen would be no safe evidence that the sugar had not been, in part at least, in a non-diffusible state. They have accordingly dialyzed blood against varying percentages of dextrose dissolved in saline and have found that the sugar con-

[2] Schenck, F., *Arch. ges. Physiol.*, 1899, xlvii, 621. Arthus, M., *Z. Biol.*, 1896, xxxiv, 432. Asher, L., and Rosenfeld, R., *Biochem. Z.*, 1907, iii, 335. Edie, E. S., and Spence, D., *Biochem. J.*, 1907, ii, 103. von Hess, C. L., and McGuigan, H., *J. Pharmacol. and Exp. Therap.*, 1914–15, vi, 45. For a general discussion see Allen, F. M., Studies Concerning Glycosuria and Diabetes, Boston, 1913, 284–290.

[3] Lépine, R., and Boulud, *Compt. rend. Acad.*, 1906, cxliii, 539. Lépine, R., Le diabète sucré, Paris, 1909, 74.

[4] Lépine, *J. physiol. et path. gén.*, 1917, xvii, 377.

[5] Michaelis, L., and Rona, P., *Biochem. Z.*, 1908, xiv, 476.

tent of the dialyzing blood remained constant only when the percentage of sugar in the saline was approximately equal to that in the blood. This is interpreted by them as indicating that the normal blood sugar is free.

EXPERIMENTAL.

Preliminary experiments with diabetic blood showed that determinations of sugar before and after a 24 hour dialysis gave no clearer evidence than the similar investigations mentioned above with normal blood. Diabetic blood and normal blood with added dextrose, when dialyzed against Ringer's solution for 24 hours in the cold, showed practically the same proportionate loss of sugar. It was then decided to use a shorter period and to take samples at frequent intervals. In the first experiments in which this was done artificial parchment thimbles were used. The results were very striking but a change had to be made in the type of dialysis membrane because the thimbles soon became difficult to obtain and, when obtained, were often found to be useless because of leaks. After considerable search a suitable type of membrane was found in the prepared mucosa of the sheep's cecum, used commercially for capping bottles of toilet preparations, etc. The various membranes of a single lot are fairly uniform in thickness and size, and in every case recorded below, the two or more dialyses, constituting a complete experiment, were performed with membranes of the same lot. All of the experiments about to be described were performed with this type of animal parchment membrane.

Method.

Experimental diabetes was produced in dogs by total or nearly total[6] depancreatization. Blood was drawn from a cannula in a femoral artery or, usually, from a jugular vein either by cannula or by aspiration through a needle. When cannulas were introduced local anesthesia was used. The blood letting was done from 2 to 16 days after the operation. The blood was allowed to flow into a dry bottle or test-tube containing a weighed amount of dry hirudin, usually 8 mg. of hirudin for about 20 cc. of blood.

[6] Allen, Studies Concerning Glycosuria and Diabetes, Boston, 1913, 476.

After stoppering, this was gently shaken for a few minutes. 15 cc. were then transferred to the dialyzing membrane. The latter had previously been soaking in Ringer's solution, after which the excess fluid was wiped off of both sides with filter paper, and it was then affixed to a suitable glass tube mounted in a clamp on a stand. The dialyzing membrane, containing the blood, was then immersed in 1 liter of Ringer's solution,[7] until the surfaces of the blood and the Ringer's solution were at the same level. A 2 cc. sample was taken at this time for analysis from the residue of the 20 cc. of blood, and at hourly intervals from the dialyzing blood. Before each sampling the temperature of the outside fluid was observed and the blood was mixed as well as possible. After sampling, the dialyzing bag was adjusted so that the surfaces of the fluids would again be at the same level. The Lewis-Benedict method, as modified by Myers and Bailey,[8] was used for the blood sugar estimations. In many of the experiments the system was kept at a low temperature by surrounding the jar with chopped ice and salt. In some, powdered thymol was added to both fluids.

As controls, normal dog blood was used. This was obtained and treated in exactly the same manner as the diabetic blood, except that weighed amounts of anhydrous dextrose were added in order to get a sugar content approximating the particular diabetic blood which it was desired to control. These controls were often shaken longer than the diabetic in order to be sure of solution of the dextrose; some even in a shaking machine to accomplish this. The control experiment was usually performed on the day after the diabetic and, of course, the attempt was made to have all experimental conditions the same.

RESULTS.

Chart I shows the types of curves which can be obtained when the blood is dialyzed against plain Ringer's solution. The diabetic

[7] The Ringer's solution used had the following composition:

	per cent
$NaHCO_3$	0.020
$CaCl_2$	0.022
KCl	0.030
NaCl	0.800

[8] Myers, V. C., and Bailey, C. V., *J. Biol. Chem.*, 1916, xxiv, 147.

blood sugar (Experiment D8′ and D8″) dialyzes slightly slower than the control (Experiment D10′ and D10″) during the 1st hour and very much slower during the 2nd hour. The 3rd and

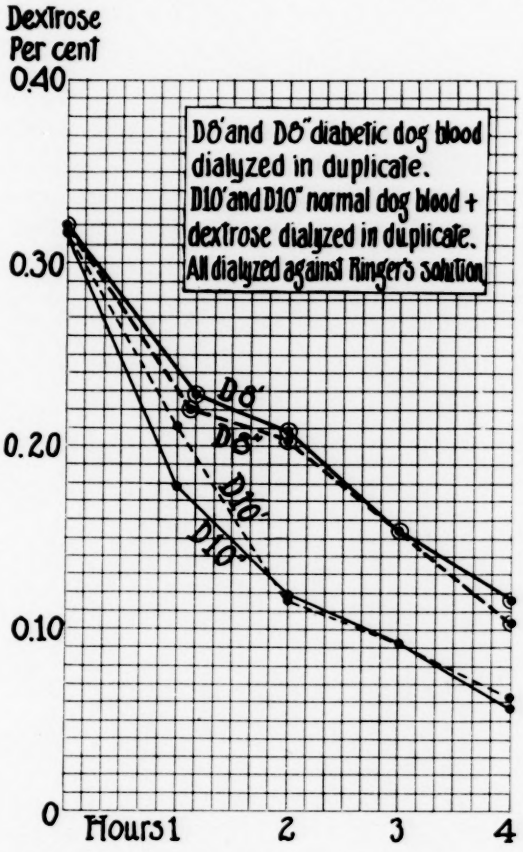

CHART I. Comparison of rate of dialysis of diabetic blood sugar with that of glucose dissolved in normal blood, using unmodified Ringer's solution. Arterial blood used. Room temperature: Experiment D8′ and D8″, 20.5–23.2°; Experiment D10′ and D10″, 20.0–22.2°. No thymol used. Diabetic blood from dog 2 days after complete pancreatectomy.

4th hours show a resumption of rapid dialysis on the part of the diabetic blood sugar. The control has a more rapid and more

regular rate throughout, but the most marked difference is in the 2nd hour in which the diabetic blood sugar dialysis is greatly retarded. It must be said, however, that such a marked differ-

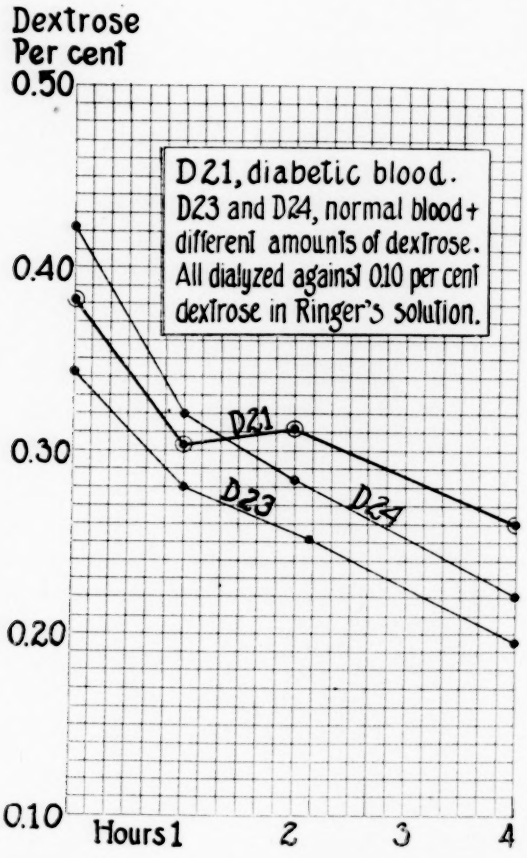

CHART II. Comparison of rate of dialysis of diabetic blood sugar with that of glucose dissolved in normal blood, dialyzed against Ringer's solution containing 0.10 per cent dextrose. Venous blood used. Temperature: Experiment 21, 4.0–13.5°; Experiments 23 and 24, 3.0–12.6°. Powdered thymol added to blood and Ringer's solution in each case. Diabetic blood from dog 16 days after 95 per cent of the pancreas had been removed (Allen method).

ence is not always obtained when unmodified Ringer's solution is used. Nevertheless this was quite similar to the type of curve which had been obtained in the above mentioned experiments in which artificial parchment thimbles had been employed. It was therefore decided to change the experimental conditions in order to accentuate the difference between the diabetic blood and the

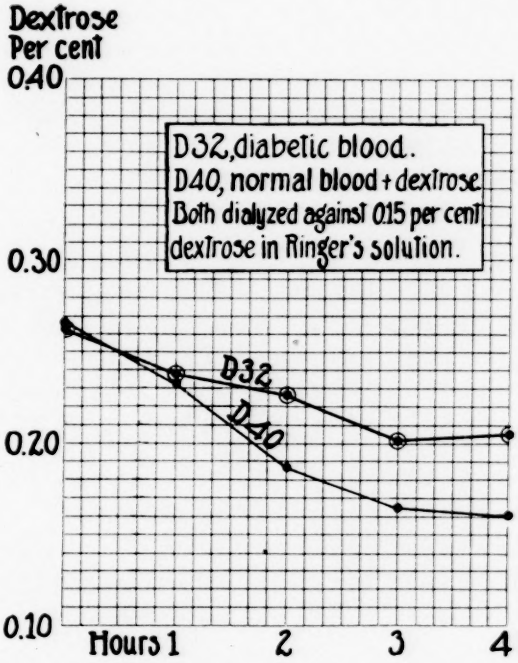

CHART III. Comparison of rate of dialysis of diabetic blood sugar with that of glucose dissolved in normal blood, dialyzed against Ringer's solution containing 0.15 per cent dextrose. Venous blood used. Temperature: Experiment 32, 1.3–2.0°; Experiment 40, 1.0–5.0°. No thymol used. Diabetic blood from dog 7 days after 95 per cent of the pancreas had been removed (Allen method).

control, if indeed any difference really existed. The fact that the animal membranes were much thinner than the artificial ones and permitted a more rapid dialysis, led to the suspicion that the production of a slightly slower rate of dialysis might accomplish this object. For this purpose a definite quantity of dextrose

was added to the Ringer's solution in both the diabetic blood dialysis and the control. This modification of the procedure resulted in a retarded dialysis and was successful in bringing out very striking differences almost invariably.

The curves in Chart II tend plainly more to the horizontal than those of Chart I, indicating, in general, a slower rate. Here the Ringer's fluid contained 0.10 per cent dextrose. The two control curves show a continuous dialysis throughout the 4 hours. In the diabetic curve the dialysis runs parallel with the controls for 1 hour after which dialysis ceases completely for 1 hour. During the last 2 hours the sugar again passes out at a rate approximating that of the controls.

With a still slower rate a similar difference is observed. In Experiments 32 and 40 (Chart III) the blood was dialyzed against 0.15 per cent dextrose in Ringer's solution. Here again the control exhibits the same type of curve. The diabetic blood sugar dialyzes at the same rate as the control during the 1st and 3rd hours but is markedly retarded during the 2nd hour and does not dialyze at all during the 4th hour. This failure to dialyze during the 4th hour cannot be likened strictly to the slowing of the control curve during the same hour, because the latter has nearly reached the level of the outside fluid (0.15 per cent) while the former is still well above it. There are therefore two periods of delayed dialysis of the diabetic blood sugar in this instance.

More experiments were made with 0.10 per cent dextrose in the Ringer's solution than of any other type. Although Chart II is typical of them, the results of a number of others are given in Charts IV and V. These experiments differ with regard to temperature and some minor points in the technique, but the same type of membrane and outside fluid was used in each case. Chart V shows the diabetic curves, for which the curves of Chart IV served as controls. Five of the six diabetic curves exhibit a delay in dialysis during the 2nd hour; in one of these (D 36) dialysis is not resumed at all after this period. In the sixth curve (D 34) the delay is seen in the 1st hour and a good rate of dialysis thereafter. In Chart IV a regular rate of dialysis for the normal blood with dextrose added occurs in all cases as shown by the logarithmic type of curve or by nearly straight lines.

The average figures for Charts IV and V have been plotted and

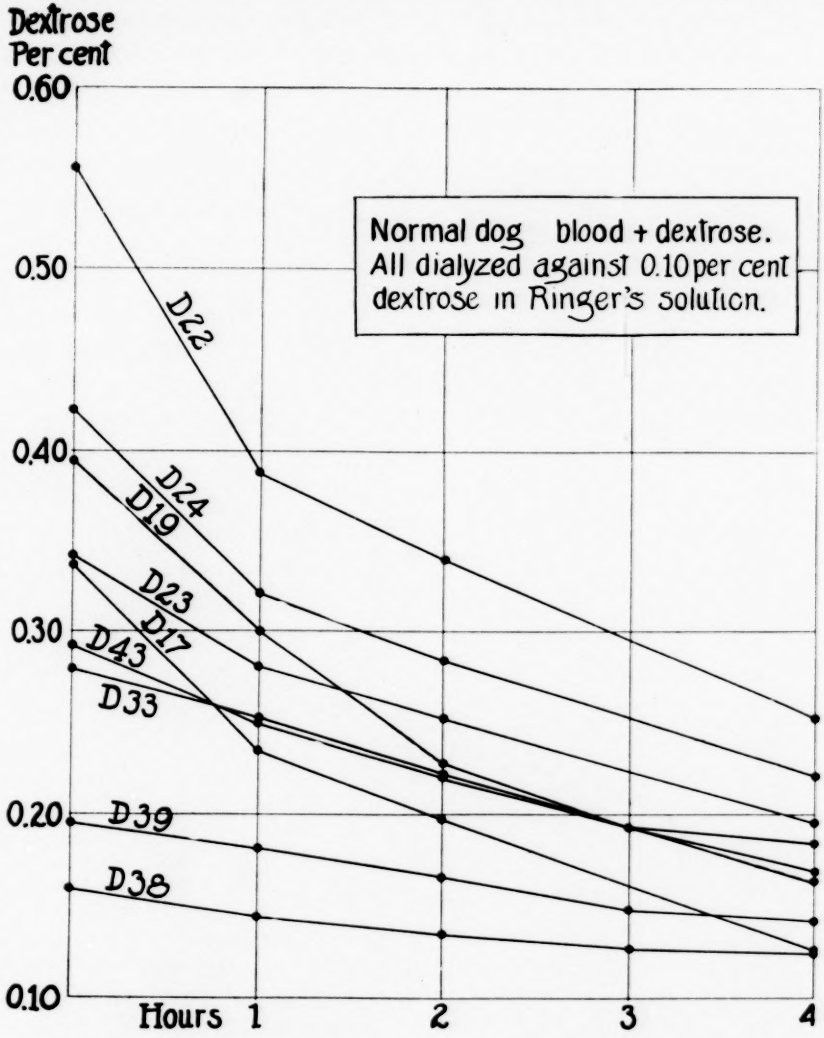

CHART IV. Dialysis curves of various specimens of normal blood with added dextrose, the temperatures varying, but all dialyzed against the same type of outside fluid.

are given in Chart VI.[3] Here the delay in the dialysis of diabetic blood sugar during the 2nd hour is clearly brought out. The control curve is a smooth, regular curve of the logarithmic type.

A few experiments have also been made with serum and with plasma.

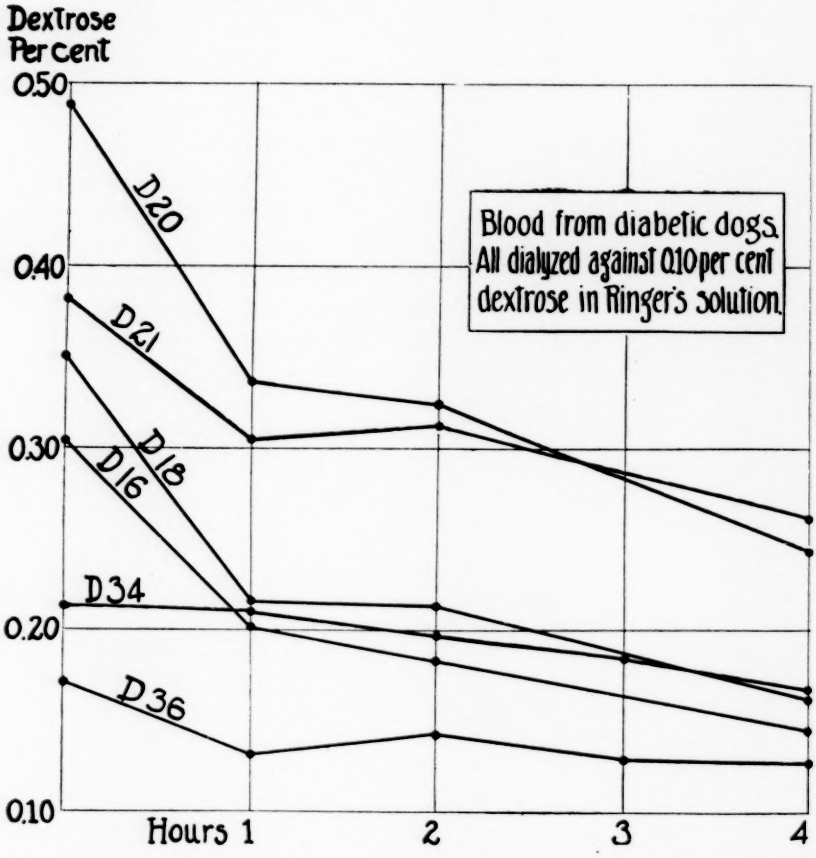

CHART V. Dialysis curves of various specimens of diabetic blood, the temperatures varying, but all dialyzed against the same type of oustide fluid.

[3] Several more experiments of this type have been performed since these curves were plotted but the results would not change the relative positions of the average curves.

Although the results were not constant they seemed to indicate that the sugar in diabetic serum does not show any appreciable difference from the control. The diabetic plasma however, exhibited a slight retardation of sugar dialysis but not as clearly as the whole blood.

DISCUSSION.

The experiments described above show unmistakably that sugar does not dialyze from diabetic blood in the same manner as glucose

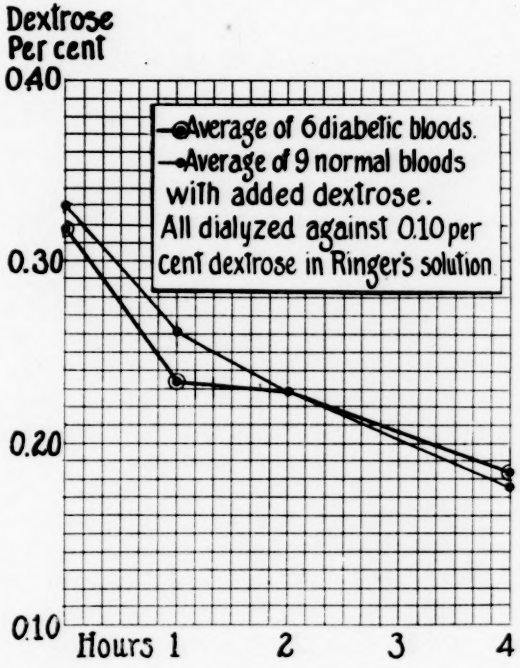

CHART VI. Dialysis curves representing the averages of the curves shown in Charts IV and V respectively.

which has been added to normal blood. There is a definite slowing, sometimes a complete cessation of dialysis at certain periods in the case of the diabetic as compared with the control. This is a definite fact and although it is easy to explain on the basis of the hypothesis which led to this investigation, several other interpretations can also be put forward.

In the first place the retardation may be due to a clogging of the membrane. It is well known, for example, that lipemia may occur after depancreatization. The fat or lipoids present in the diabetic blood may perhaps form a film on the surface of the membrane or become dissolved in the membrane and thus inhibit the passage of the sugar through it. In order to produce a result like that of Chart I, for example, we must suppose that this film forms during or near the end of the 1st hour in order to prevent the dialysis during the 2nd hour and that it falls off or disappears after the 2nd hour in order to permit of the free dialysis which occurs later. Furthermore, it would have to form again in order to give us the second delay seen in Chart III. Such changes, while not impossible, would seem to be unlikely.

There is also the possibility that these delays in dialysis are not delays at all but that the higher values obtained are due to a formation of sugar while the dialysis continues. In many of the experiments a sample of the blood was kept *in vitro* at the same temperature as the dialysis and during the same period. In no case did an increase in sugar content occur; in fact there was usually a slight decrease even at low temperatures. This is indeed no absolute proof that new sugar is not produced in the dialyzing specimen for here we might conceive of the removal, by dialysis, of substances which inhibit such production of sugar. In some of the experiments with diabetic blood there have been seen slight increases in sugar content in the course of the dialysis (see Charts II and V) but whether this is a real increase or is an error due partly to insufficient mixing before sampling, cannot be said. Lépine and Boulud[9] found that after dialyzing fresh serum against saline for 2 hours the sugar content of the serum was often greater than that of the control kept at the same temperature. They explained this by assuming that a certain amount of water had passed through the membrane into the blood and had caused the formation there of dextrose from the *sucre virtuel*. Furthermore, Milne and Peters,[10] as well as Myers and Killian[11] have shown that in depancreatized dogs the blood diastase is often greatly increased

[10] Milne, L. S., and Peters, H. LeB., *J. Med. Research*, 1912, xxvi, 415.
[11] Myers, V. C., and Killian, J. A., *J. Biol. Chem.*, 1917, xxix, 179.

in amount. The formation of new sugar during the course of dialysis would seem then to be possible, and, from the evidence, more probable than an effect upon the membrane. However, the phenomenon described occurs to just as marked an extent—if not to a more marked extent—in the cold as at higher temperatures, a fact which would be inconsistent with an enzyme hypothesis. Moreover another one of the facts mentioned above cannot be explained on the basis of this hypothesis. This fact is that when the blood is dialyzed against unmodified Ringer's solution the difference between the diabetic blood sugar dialysis and the control is sometimes not well marked. If new sugar is being formed to any great extent it should, of course, regularly appear as an apparently retarded dialysis. In other words, the more rapid dialysis in this series, caused by a greater difference in osmotic pressure, should not have prevented a new formation of sugar. However, it did prevent the regular occurrence of a notable difference between the diabetic and control curves and this would seem to indicate that a new formation of sugar is not the chief cause of this phenomenon.

A third hypothesis is that part of the blood sugar in experimental diabetes is in a combined state. The type of combining substance or manner of combination need not be discussed, for such discussion would be mere speculation. According to this idea sugar is readily broken off from its combination by most chemical procedures; *e.g.*, as in the removal of the protein from the blood prior to sugar determination. Therefore the determination of blood sugar at any time during the dialysis reveals the amount of free and combined sugar present at that time. The combination is also easily split, probably, by physical means, such as the clotting of the blood, centrifugalization, and perhaps also by the action of an excess of water. Combined sugar, being a much larger molecule than free or crystalloid sugar, presumably does not pass through the membrane while crystalloid glucose easily does. If all the sugar in the blood were combined the dialysis curve would be a horizontal line, indicating no dialysis, until, from one cause or another, the combination would begin to break down, at which time the sugar liberated would begin to dialyze. If all the sugar is free the curves will be such as are seen in the controls of the

present investigation.[12] The curves obtained with diabetic blood seem to indicate that the sugar is partly free and partly combined. It is true that it would be difficult to predict the exact form of curve which would delineate the dialysis of such a mixture. This is particularly true if the combined sugar is not in a stable union and may disintegrate either gradually or suddenly if the equilibrium between free and combined sugar is disturbed.

However, the curves obtained with diabetic blood lend themselves easily to interpretation from this view point. A large amount of free sugar—depending upon the proportion which is balanced by any sugar in the outside fluid—passes through the membrane first. The delay, which the curves show, whether in the 1st, 2nd, 3rd, or 4th hour, indicating as it does that dialysis is not continuing, means that there is now present a comparatively large proportion of combined sugar which cannot pass through the membrane. The resumption of dialysis is caused by a disintegration of the sugar combination, liberating free sugar which, of course, dialyzes. When there are two periods of retarded dialysis, as in Chart III (Experiment D 32), it would mean either that there were two types of combined sugar or that equilibrium was adjusted a second time.

The fact that with plain Ringer's solution the difference between the diabetic and control curves is not always sharp can be explained on the basis of this hypothesis as a sudden physical cleavage of the combined sugar molecule due to the extremely rapid dialysis, with a rapid upset of the equilibrium between free and combined sugar. With slower dialysis, produced by a thicker membrane, as in the preliminary experiments, or by decreasing the difference in osmotic pressure by adding dextrose to the Ringer's solution, the peculiar unevenly retarded curve has always been obtained with diabetic blood.

There is only one point which is difficult to explain by the combined sugar hypothesis; namely, the slight increase in the sugar which at times is seen in the course of the dialysis of a diabetic blood. If this increase is real, that is, if it is not an error of

[12] The question of the occurrence of combined sugar in the normal blood of these controls may be disregarded for the purpose of this discussion because of the relatively large amount of crystalloid glucose added.

method or of sampling, there are two possible explanations, neither of which is entirely satisfactory. The first is that there may also be present a certain amount of another type of combined sugar which is precipitable by the protein precipitant (picric acid); and it is only when this combination disintegrates (and hence is not precipitated by the picric acid) that its sugar appears in the analytical result as an apparent increase in sugar content. If this explanation is not acceptable then we must agree that there is active here, at the same time, a new formation of sugar to a slight degree. The possibility of a new formation of sugar as the explanation of the entire phenomenon has been discussed above as well as the reasons for considering this improbable. However, it may occur to a slight extent simultaneously with a delay in dialysis of combined sugar and thus give rise to the type of curve which has not only a delay but an increase in the blood sugar.

Significance.—Diabetes is a very complicated phenomenon and any new fact which can be established concerning it may aid in its explanation. The experiments described above indicate that *in experimental diabetes there exists some mechanism which tends to hinder the dialysis of the blood sugar.* Assuming that the same mechanism operates in the organism, can this fact aid in explaining diabetes?

For the production of hyperglycemia the presence of substances which can combine with glucose may, indeed, play some rôle. Let us suppose that such substances, whether toxic or not, are produced by the normal organism and are destroyed or neutralized if the pancreas is functioning in a normal manner. When the pancreas is removed, these substances circulate in the blood and combine with the dextrose. This leaves a dearth of free sugar in the blood and to remedy this condition the liver or muscles convert some of their glycogen store into dextrose. Thus we may have a hyperglycemia with varying proportions of free and combined sugar. It is also possible that the "combining substance" is toxic and that there is an outpouring of sugar in order to combine with and render it non-toxic; in other words this would be a protective action. However, whether primarily due to a sugar combination or to some other cause, hyperglycemia occurs. Why should hyperglycemia be maintained and why should the animal gradually lose its strength and waste away?

As a large proportion of the blood sugar is bound in the form of a colloidal molecule it cannot pass through the capillary walls, or does so with difficulty. Consequently it cannot reach the tissues and be utilized. It remains in the blood and thus the high blood sugar is maintained, with usually a glycosuria as well. Moreover, the proportion of free dialyzable sugar is at the same time probably less than normal, and therefore, if other conditions remained constant there would be less free sugar available for the tissues, which would slowly starve. But the other conditions are not the same even for free sugar, for Kleiner and Meltzer[13] have shown that when large quantities of glucose are injected intravenously, they pass into the tissues very slowly as compared with the normal, indicating a decreased permeability of the endothelium of the capillaries in diabetes. Therefore two factors tend to prevent the tissues from obtaining a supply of sugar; *viz.*, the conversion of a large proportion of the sugar into a difficultly diffusible form and the decrease in the permeability of the endothelium of the capillaries even to free sugar. The tissues in experimental diabetes apparently do not lack the normal capacity to burn or store sugar.[10, 14] The fault is not that the tissues do not handle sugar well but that they cannot get the sugar. Palmer[15] has shown that the striated muscle of diabetic animals has a lower concentration of dextrose than that of normal animals when the levels of blood sugar are taken into consideration. This fact tends to support the contention that, for one reason or another, the sugar does not get into diabetic tissues. Therefore, since the tissues are not properly nourished the animal gradually loses strength and ultimately dies.

The possibility that glucose combines with some substance or substances in the body has been suggested by various investigators. Several[16] have believed that such a combination is

[13] Kleiner, I. S., and Meltzer, S. J., *Proc. Soc. Exp. Biol. and Med.*, 1914–15, xii, 58; *Proc. Nat. Acad. Sc.*, 1915, i, 338.

[14] Cohnheim, O., *Z. physiol. Chem.*, 1903, xxxix, 341; 1906, xlvii, 253.

[15] Palmer, W. W., *J. Biol. Chem.*, 1917, xxx, 79.

[16] Pavy, F. W., On Carbohydrate Metabolism, London, 1906, 9, 33, 71, 111. Eppinger, H., and Falk, F., *Berl. klin. Woch.*, 1911, xlviii, 1625. Allen, Studies Concerning Glycosuria and Diabetes, Boston, 1913, 402. Ringer, A. I., *J. Biol. Chem.*, 1914, xvii, 107.

a prerequisite for sugar utilization. Others[17] have thought that this combination might be of such a nature as to resist oxidation. The present hypothesis, which is merely an hypothesis on the basis of these dialysis experiments, presents a totally different conception; namely, that a considerable part of diabetic blood sugar, being in a combined and less easily diffusible condition, is not stored or used by the cells because it cannot pass from the blood through the walls of the capillaries and into the tissues.

The glycosuria of diabetes need not concern us in this discussion. It presents nothing which is incompatible with the present hypothesis, for, as stated above, the kidney is able to secrete colloids as well as crystalloids. Other questions in regard to the general problem have also been omitted for it is not to be expected that the observations recorded here will explain the entire question of diabetes. It is hoped, rather, that they will fit in with other facts and thus aid in its interpretation.

SUMMARY.

Blood from diabetic dogs was dialyzed against Ringer's solution, to which was usually added a small amount of dextrose. The rate of dialysis of the blood sugar was determined by analyzing samples taken at regular intervals. This was compared with similar dialyses of normal dog blood which had been brought to the same sugar content by the addition of dextrose. The diabetic blood sugar dialyzed at an irregular rate, with a delayed or completely interrupted dialysis during one or more periods, usually the 2nd hour. The control dialysis (normal blood with added sugar) was not interrupted in this manner.

This is interpreted as possible evidence for the existence of "combined" sugar in diabetic blood, the significance of which is discussed.

[17] Minkowski, O., *Arch. exp. Path. u. Pharmakol.*, 1892-93, xxxi, 179. Stiles, P. G., and Lusk, G., *Am. J. Physiol.*, 1903-04, x, 78.

THE INFLUENCE OF NEUTRAL SALTS, BASES, AND ACIDS ON THE PRECIPITABILITY OF GELATIN BY ALCOHOL.

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I.

It has been shown in the previous publications[1] that the effect of neutral salts on gelatin is repressed in the presence of the salt and that we can study such effects with any profit only when the excess of salt is washed away after it has had a chance to act on the protein. Such gelatin reveals qualities which are masked in the presence of the salt and which allow us to gain a clear insight into the effect of the salt. The first striking result was that contrary to the current belief the cation of the salt was found to be the active ion, while the anion did apparently not act at all. All salts with univalent cation and bivalent anion (type Na_2SO_4 or K_2 oxalate) had exactly the same effect as all the salts of the type NaCl or NH_4Cl (univalent cation and univalent anion) when the cation concentration was the same in solutions of both salts. While the salts with univalent cation caused an additional swelling and an increase in viscosity of the gelatin after the salt was washed away, the salts with bivalent cation caused little or no such additional swelling and no or only a slight increase in viscosity.

This difference in the effect of univalent and bivalent cations, as well as the repressing effect of the presence of the salt, was explained on the assumption that the neutral salts form with gelatin metal gelatinates which dissociate electrolytically into a negative gelatin ion and a positive cation (that of the salt used), while the anion either does not combine at all with the gelatin

[1] Loeb, J., *J. Biol. Chem.*, 1917, xxxi, 343; 1918, xxxiii, 531; xxxiv, 77, 395.

or forms a non- or less dissociable compound. Univalent cations, like Na, form gelatinates which are highly dissociable electrolytically, while the bivalent metals form non- or less dissociable compounds. The presence of the salt represses the electrolytic dissociation of the metal proteinate formed and this repressing effect makes it necessary to remove the excess of salt if we wish to study the effect of the salt on the protein, since many of these effects are due to the ionizing action of the salt on the protein as a consequence of the formation of metal proteinates.

It was pointed out that these results lead to a considerable simplification of protein chemistry. According to our results, gelatin behaves like a weak acid, which, if in contact with a neutral salt, will exchange some of its H ions for the cations of the salt. The anions of the salt do not enter into combination with the gelatin or if they do their effect is not noticeable. When, however, we treat gelatin with a comparatively low concentration of a strong acid, *e.g.* M/100 HCl, gelatin chloride or hydrochloride is formed which should be able to exchange its anion when treated with a neutral salt, while the cation of the latter should not enter into any reaction with the gelatin, or if it does its effect should not be noticeable. This was found to be the case. $NaNO_3$ and $Ca(NO_3)_2$ act alike upon gelatin chloride when their NO_3 concentration is the same, and if the Ca and Na enter into combination with the gelatin chloride their influence is not noticeable. This contradicts the current statement, especially advocated by Pauli,[2] that the action of a neutral salt upon a protein is the algebraic sum of the opposite effects of the anion and cation of the salt used.

In this paper we wish to test our views further by investigating the effects of a treatment of gelatin by neutral salts upon the precipitability of the gelatin by alcohol. We have occasionally mentioned such investigations but have not yet published any systematic results.

The procedure was as follows. 1 gm. of Cooper's gelatin (grain size between sieve 50 and 60) was put into each of a series of beakers containing 100 cc. of various concentrations of a neutral salt, usually from M/1 or M/2 down to M/2048.[3] One beaker with

[2] Pauli, W., *Beitr. chem. Physiol. u. Path.*, 1903, iii, 225.

[3] If the concentration of the salt is too high it may dissolve some of the gelatin.

distilled water served as a control. The gelatin powder remained in the beaker for 1 hour and was repeatedly stirred during this time. After 60 minutes the gelatin was poured into a cylindrical funnel and the salt solution was allowed to drain off. Then each cylinder was perfused six times with 25 cc. of H_2O to wash off all the salt solution contained in the capillary spaces between the granules.[4] In the washing the additional swelling appeared which has been discussed in the previous papers. The mass of gelatin was then liquefied by heating for about 10 minutes in a water bath of 50° C., and the amount of 95 per cent alcohol was ascertained which was required to precipitate 5 cc. of a 1 per cent solution of such gelatin at about 20° C. The method here described has to be followed minutely if the same results as are described in this paper are to be obtained. Differences in the temperature and time of exposure of the gelatin to the salt solution, as well as differences in the amount of washing with distilled water, influence the results.

It is of interest to compare the quantity of alcohol required for precipitation of washed and non-washed gelatin (Table I). The sign ∞ means that the gelatin can no longer be precipitated or that no end-point can be reached with the addition of 20 cc. or more of alcohol.

The reader will notice at a glance the striking difference between the alcohol precipitability of gelatin in the presence of the salt and after the salt is washed away. In the latter case we have a striking *critical point*, which is identical for NaCl and LiCl, namely at M/128, where the gelatin suddenly becomes non-precipitable, while at the next lower concentration (M/256) comparatively little alcohol was required for precipitation.

No such critical point exists when we test the alcohol precipitability of the 1 per cent gelatin solution in the presence of the salt with which it had been treated. The quantities of alcohol required rise slowly with the concentration of the salt, but the differences are too small to select definite values as standards of comparison for the effect of different salts on the gelatin.

[4] On account of the hydrolytic dissociation of gelatin salts an excessive washing will finally lead to the formation of pure gelatin again. The washing, therefore, must be restricted to a removal of the free solution of the original electrolyte existing in the capillary spaces between the granules.

Experiments with acids and alkalies have led Pauli and his pupils to the conclusion that when the gelatin is sufficiently ionized it becomes non-precipitable by alcohol.[5] If we apply this to our experiments with neutral salts we may draw the conclusion that if gelatin is put for 1 hour into a solution of NaCl or LiCl of M/128 or above, it is ionized to such an extent that it ceases to be precipitable by alcohol, provided the excess of salt is washed away, but that if the salt is not washed away the ionization of the sodium or lithium gelatinate is repressed on account of the common ion; hence the gelatin remains precipitable in the presence of the salt.

Table I indicates still another fact worth mentioning; namely,

TABLE I.

		Cc. 95 per cent alcohol required for precipitation of 5 cc. 1 per cent solution of gelatin (at 20° C.) previously treated for 1 hour with												
		M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1024	M/2048	H_2O
In presence of salt.	NaCl	10.0	8.6	7.4	6.2	5.3	4.1	3.9	4.1	3.4	3.3	3.3	3.4	3.3
	LiCl	11.05	10.45	8.7	7.55	6.65	6.9	5.8	4.55	5.15	3.55	3.25		3.6
In absence of salt (washed six times with H_2O).	NaCl	∞	∞	∞	∞	∞	∞	∞	∞	6.6	5.8	5.45		5.4
	LiCl	∞	∞	∞	∞	∞	∞	∞	∞	7.6	6.5	6.8	5.6	5.35

that if we perfuse our powdered gelatin six times with 25 cc. of distilled water after it has been kept for 1 hour in 100 cc. of H_2O, it requires more alcohol for precipitation, namely about 5.5 cc., than when we soak it merely for 1 hour in distilled water; in the latter case it can be already precipitated by about 3.5 cc. of alcohol. The difference is due to the traces of salt which the gelatin contains in the form of impurities and which are removed by sufficient perfusion with distilled water after it has been kept for 1 hour in distilled water. These impurities repress the ionization of the gelatin.

[5] Pauli, *Fortschr. naturwiss. Forschung*, 1912, iv, 223.

The reader will notice that the washed gelatin serving as control has about the same alcohol number in all the experiments reported in this paper. This alcohol number of washed gelatin indicates that normally a certain amount of ionization of gelatin into a negative gelatin ion and a positive hydrogen ion exists since we shall see later that if we add acid to make the gelatin isoelectric a 1 per cent solution precipitates spontaneously on standing at a low temperature and little or no alcohol is required to bring the precipitation to the standard used in these experiments (see Table IV).

Fenn[6] has recently published a series of careful investigations on the influence of electrolytes upon the precipitation of gelatin by alcohol, but all his experiments as well as those of his predecessors on the same effect were made on gelatin in the presence of an excess of electrolytes used. As long as the experiments are made with very dilute acids or alkalies no serious error results, but in the case of higher concentrations of electrolytes (and in the case of salts higher concentrations are required) this method must give ambiguous results since the ionization of the gelatin will be repressed by the presence of the electrolyte. In our experiments this excess of electrolytes was washed away after they had had a chance to act on the gelatin and our experiments have led to entirely different results (Table II).

The reader will see from Table II that under our method of procedure all salts with univalent cation and univalent anion (type NaCl) render the gelatin solution non-precipitable in concentration of M/128 or slightly above (after the salt is washed away). For the majority of cases the critical concentration was M/128.

All salts with univalent cation and bivalent anion (type Na_2SO_4) make the gelatin solution non-precipitable with alcohol at exactly half the molecular concentration of salts of the type NaCl; namely, M/256 or slightly above. For all with the exception of one salt the value was M/256. This proves that the effect of the salt is determined exclusively by the cation and that the anion does apparently not influence the effect.

When gelatin is treated with neutral salts of the alkali earth

[6] Fenn, W. O., *J. Biol. Chem.*, 1918, xxxiii, 279, 439; xxxiv, 141, 415.

TABLE II.

Type of salt.	Nature of salt.	Cc. 95 per cent alcohol required for precipitation of 5 cc. 1 per cent solution of gelatin (at about 20° C.) previously treated for 1 hour with 100 cc. of one of the following solutions and then freed from the excess of salt by perfusing six times with 25 cc. H_2O.												
		M/1	M/2	M/4	M/8	M/16	M/32	M/64	M/128	M/256	M/512	M/1024	M/2048	H_2O
I. Univalent cation, univalent anion.	NaCl	∞	∞	∞	∞	∞	∞	∞	∞	6.6	5.8	5.45		5.4
	KCNS				∞	∞	∞	∞	∞	6.8	6.0	5.6		5.5
	LiBr	∞	∞	∞	∞	∞	∞	∞	∞	7.3	6.2	6.0		5.7
	NH_4Cl	∞	∞	∞	∞	∞	∞	∞	∞	9.6	6.0	5.5	5.5	5.35
	LiCl	∞	∞	∞	∞	∞	∞	∞	∞	7.6	6.5	6.8	5.6	5.35
	KCl			∞	∞	∞	∞	∞	8.6	6.2	4.8	5.1		5.0
	$LiNO_3$			∞	∞	∞	∞	∞	9.8	6.2	5.8	5.4		5.45
	Na acetate	∞	∞	∞	∞	∞	∞	∞	12.4	7.15	5.8	5.7	5.4	5.6
II. Univalent cation, bivalent anion.	Na_2SO_4		∞	∞	∞	∞	∞	∞	∞	∞	7.5	5.5		5.5
	Na_2 oxalate				∞	∞	∞	∞	∞	∞	∞	6.8	6.3	5.85
	Na_2 tartrate	∞	∞	∞	∞	∞	∞	∞	∞	∞	8.0	6.6		5.7
	Na_2 succinate	∞	∞	∞	∞	∞	∞	∞	∞	∞	8.1	6.15	5.8	5.5
	K_2SO_4			∞	∞	∞	∞	∞	∞	∞	6.85	6.0	5.7	5.45
	Li_2SO_4			∞	∞	∞	∞	∞	∞	8.0	6.6	5.3		4.8
III. Bivalent cation, univalent anion.	$MgCl_2$	6.2	6.3	7.05	7.25	7.6	7.4	7.4	6.2	6.7	5.3	5.8	5.6	5.4
	$CaCl_2$			5.1	4.9	5.0	5.3	5.0	5.1	5.3	5.3	5.4	5.4	5.4
	$SrCl_2$		5.3	4.95	5.2	5.6	6.0	5.8	5.5	5.5	5.5	5.5	5.4	5.4
	$BaCl_2$			4.45	4.8	5.6	4.1	4.7	4.6	4.3	5.0	5.2	6.0	5.6
IV. Bivalent cation, bivalent anion.	$MgSO_4$		6.3	6.5	6.3	6.4	6.6	6.6	6.4	6.4	5.5	5.5	5.1	5.1	5.1

metals the gelatin remains precipitable with alcohol after the salt is washed away no matter in what concentration the salt had been used.

A study of the alcohol numbers of the gelatin first treated with the salts of alkali earth metals and then freed from the excess of salt by washing with distilled water shows that only after the treatment with $MgCl_2$ and $MgSO_4$ does the alcohol number rise slightly beyond that of gelatin washed with water but not previously treated with salt (under H_2O in Table II). Hence Mg is the only metal of this group which leads to a slight increase in ionization. It is also worth noticing that $MgCl_2$ and $MgSO_4$ act quantitatively alike, thus supporting our statement that the influence of the anion of the neutral salt does not make itself felt in these experiments.

These experiments on alcohol precipitation thus confirm the conclusion, drawn from our experiments on swelling and viscosity, that only the cation of the neutral salts influences the gelatin and that there is a typical difference in the action of univalent and bivalent cations.

The results can be best explained on the assumption that the neutral salts form with gelatin (and perhaps with all proteins which are stronger as acid than as base) metal proteinates, which are highly ionizable when the metal is univalent (Na, Li, K, NH_4) and much less ionizable when the salt used is bivalent (Mg, Ca, Sr, Ba).

II.

If this assumption is correct it follows that treatment of gelatin with solutions of NaOH or KOH should act exactly like treatment with solutions of neutral salts, leading to the production of highly ionizable Na or K gelatinate no longer precipitable with alcohol, and that this ionization should become manifest after the excess of the alkali has been washed away by the repeated perfusion of the powdered gelatin with distilled water.

It would also follow that treatment of the gelatin with $Ca(OH)_2$ or $Ba(OH)_2$ should act like the treatment of gelatin with $CaCl_2$ and $BaCl_2$; *i.e.*, after the base is washed away, the gelatin should remain precipitable no matter what the concentration of the

$Ca(OH)_2$ or $Ba(OH)_2$.[7] These demands of the theory are fulfilled (Table III). 1 gm. of the finely powdered gelatin was put for half an hour at about 22°C. into 100 cc. of various concentrations of NaOH, KOH, $Ca(OH)_2$, and $Ba(OH)_2$. The gelatin was then put into a funnel, the alkali allowed to drain off, and the gelatin was then perfused four times with 25 cc. of distilled water. After this the gelatin was liquefied and the precipitability of 5 cc. of 1 per cent solution by 95 per cent alcohol was determined at about 20°C. Gelatin which had been previously treated with M/256 or still more dilute KOH or NaOH was precipitable with alcohol, while gelatin treated with M/128 or higher concentrations of these two bases was no longer precipitable with alcohol, after

TABLE III.

Nature of base	Cc. 95 per cent alcohol required for precipitation of 5 cc. 1 per cent solution of gelatin, at 20°C., previously treated for half an hour with one of the following solutions and then perfused four times with 25 cc. H_2O.								
	M/16	M/32	M/64	M/128	M/256	M/512	M/1024	M/2048	H_2O
KOH	∞	∞	∞	∞	15.9	7.55	6.4	5.25	5.35
NaOH	∞	∞	∞	∞	8.6	6.8	6.2	5.5	5.7
$Ca(OH)_2$			4.4	5.5	5.3	5.3	5.3	5.9	5.8
$Ba(OH)_2$		4.3	4.0	4.0	4.3	4.3	5.0	5.4	5.3

the excess of base was washed away. The critical concentration is therefore under the conditions of our experiments the same for NaOH and KOH as for NaCl and KCl.

On the other hand, gelatin treated with weak or strong concentrations of $Ca(OH)_2$ or $Ba(OH)_2$ did not lose its alcohol precipitability but had the same low alcohol number as gelatin treated with salts of Ca or Ba. No more striking proof of our theory could be expected.

The fact that the alkalies give the same results as the neutral salts confirms our view that the salts combine with the gelatin, forming metal gelatinates, since the combination of alkalies with gelatin can be proved by titration experiments.

[7] The concentrations in which bases can be used are limited by the fact that higher concentrations liquefy the gelatin.

III.

Experiments with acids do not contradict our theory but the agreement is not so perfect as in the case of alkalies. According to our theory all strong monobasic acids should render the gelatin non-precipitable at the same concentration (after the acid is washed away). This seems to be the case, the critical concentration being in the neighborhood of M/256 for HCl, HNO_3, and trichloroacetic acid (Table IV).

The dibasic acids, like H_2SO_4, should, however, act like diacidic bases, *e.g.* $Ca(OH)_2$, and should not annihilate the alcohol

TABLE IV.

Nature of acid.	Cc. 95 per cent alcohol required for precipitation of 5 cc. 1 per cent solution of gelatin, at 20°C., previously treated for half an hour with one of the following solutions and then perfused four times with 25 cc. H_2O.										
	M 4	M 8	M 16	M 32	M 64	M 128	M 256	M 512	M 1024	M 2048	H_2O
HNO_3		∞	∞	∞	∞	∞	∞	0.7	3.7	4.7	5.1
HCl			∞	∞	∞	∞	∞	1.1	4.5		5.3
Trichloroacetic acid	∞	∞	∞	∞	∞	∞	∞	0.95	3.7	4.95	5.4
H_2SO_4				∞	17.0	12.2	10.2	7.1	1.1	4.0	5.5
Tartaric acid	∞	∞	∞	∞	∞	∞	∞	6.15	0.9	4.15	5.65
Oxalic acid	∞	∞	∞	∞	∞	∞	∞	8.75	1.0	3.4	5.05
Malic acid	∞	∞	∞	∞	∞	∞	6.8	1.4	1.4	4.3	5.3

precipitability of gelatin or at least not at low concentrations of the acid. This is true only for H_2SO_4, and here only partly, but not for the organic dibasic acids, like tartaric, oxalic, and malic. These organic dibasic acids behaved like the monobasic acids (Table IV). The reason for this deviation is not clear. Should it be that the organic acids, like oxalic or tartaric acid, and to a lesser extent also H_2SO_4, form acid salts with gelatin?

The experiments on acids give us an opportunity to compare our results with those of colloid chemists, especially Pauli. Pauli investigated the effects of acid on proteins but in the presence of the acid. As long as low concentrations of acids are used the error is not so great, but with higher concentrations erroneous

results are unavoidable. Thus Pauli[5] states that if acids are arranged according to their power of influencing in moderate concentrations viscosity and precipitability with alcohol they must be grouped in the following order: trichloroacetic, dichloroacetic, sulfuric, nitric, hydrochloric, monochloroacetic, and acetic acid. A comparison of this statement with the results on gelatin after the excess of acid is washed away (Table IV) shows that Pauli's statement is no longer tenable.

It might be well to point out that our experiments with acids support in another respect the ionization hypothesis very nicely. In these experiments the gelatin was first treated for half an hour at about 20° with 100 cc. of acid and the latter was then washed away by four perfusions with 25 cc. of H_2O each, as in the experiments with alkalies. The reader will notice that before the gelatin becomes non-precipitable by alcohol it goes through a stage where it can be precipitated with less alcohol than is required for the gelatin not treated with acid. This is due to the fact that gelatin is a stronger acid than base and hence dissociates slightly into H^+ and gelatin. Since gelatin is only a weak acid it requires the addition of only a trace of a second acid to repress this ionization, and for gelatin treated with HCl and then washed four times with 25 cc. of H_2O this point—the isoelectric point—is reached when the acid used was M 512 (Table IV). It was the same for nitric and trichloroacetic acid. For dibasic acids the isoelectric point was at twice the dilution of that in the case of monobasic acids; namely, M 1024 (Table IV). If more acid is added, a salt formation between acid and gelatin is produced, the acid combining with a NH_2 group of the gelatin, the gelatin salt undergoing stronger ionization. This action of the acid had been noticed and correctly interpreted by Pauli.[5] This observation supports the assumption that the non-precipitability of gelatin treated with electrolytes, acids, alkalies, and neutral salts, is due to ionization of the gelatin.

The fact that the isoelectric point is reached at the same hydrogen ion concentration regardless of the nature of the anion of the acid shows that only the former ion influences the result. Exactly the same is true as far as the alcohol precipitability of

[5] Pauli, *Arch. ges. Physiol.*, 1910, cxxxvi, 483.

gelatin by different monobasic acids is concerned, as our Table IV shows.

The writer wishes to call attention to the fact that a 1 per cent gelatin solution near the isoelectric point becomes on standing at not too high a temperature turbid or completely opaque even if no alcohol is added. This only happens when the gelatin has been treated with acid but not with base or with a neutral salt.[9] Such naturally turbid isoelectric solutions of gelatin are also characterized by a minimum in viscosity, and a minimum in swelling, all of which tends to support the conclusion that these physical qualities are a function of the degree of ionization of the gelatin.

IV.

Weak acids and bases, *e.g.* acetic acid and NH_4OH, will not cause the gelatin to become non-precipitable. 1 gm. of gelatin

TABLE V.

	Cc. 95 per cent alcohol required for precipitation of 5 cc. 1 per cent solution of gelatin, at 20°C., previously treated for 1 hour with one of the following solutions and then perfused four times with 25 cc. H_2O.										
	M 4	M 8	M 16	M 32	M 64	M 128	M 256	M 512	M 1024	M 2048	H_2O
Acetic acid	6.1	5.5	4.9	4.3	1.8	1.0	0	1.4	3.8	4.7	5.1
NH_4OH	13.5	8.1	6.3	5.9	5.7	5.8	5.5	5.6	5.5		5.4

was put for 1 hour into beakers containing 100 cc. of different concentrations of these two substances and was afterwards freed from the excess of the solution by four perfusions with 25 cc. of distilled water, as described. It was found that the gelatin so treated remained precipitable by alcohol in all the concentrations used (Table V).

It is noticeable that the values for acetic acid go through a minimum at M/256 while those for NH_4OH do not show such a minimum, as was to be expected.[9]

[9] It is impossible to bring gelatin nearer the isoelectric point with the aid of salts or bases. For amphoteric electrolytes which are stronger acids than bases the isoelectric point is reached when the dissociation of the H ions is repressed to the level of that of the OH ions, and such a result can only be brought about by the addition of acid to the gelatin solution, but not by the addition of a neutral salt. It is, therefore, not correct to state that a salt like $CaCl_2$ or NaCl can render a protein like gelatin "isoelectric."

The slight effect of acetic acid is not a specific effect of the acetate anion but merely due to the fact that acetic acid is a weak acid, since the weak base NH_4OH shares this peculiarity of the acetic acid. This effect of weak acid and base is a confirmation of our theory, since we assume that electrolytes react in a purely chemical way with gelatin (and proteins in general). Gelatin being a weak acid and a still weaker base, only little gelatin acetate and ammonium gelatinate can be formed with acetic acid and NH_4OH respectively. The mass of the new gelatin salt being small at the best, not as many gelatin ions can be formed as are required to render the gelatin non-precipitable by alcohol.

These negative effects of the treatment of gelatin with weak acid and weak base (after the excess of acid or base is washed away with distilled water) are paralleled by equally negative effects on additional swelling and on viscosity.

SUMMARY.

1. The experiments reported in this paper show that if finely powdered gelatin has been treated for 1 hour with the solution of a neutral salt of the type NaCl (univalent cation, univalent anion), and if the excess of salt solution is afterwards washed away with H_2O, a 1 per cent solution of such gelatin in distilled water becomes non-precipitable by alcohol when the concentration of the salt solution used exceeds a certain limit, which is about M/128.

2. When the neutral salt used is of the type Na_2SO_4 (univalent cation, bivalent anion) the critical molecular concentration which renders the 1 per cent gelatin solution under the conditions of our experiments non-precipitable by alcohol is exactly one-half of that for salts of type NaCl; namely, about M/256.

3. When the powdered gelatin is treated with solutions of neutral salts of the type $CaCl_2$ or $MgSO_4$ (*i.e.* bivalent cation, univalent or bivalent anion) and the excess of salt is washed away the 1 per cent gelatin solution in distilled water remains precipitable with alcohol for all concentrations of the salt used.

4. These experiments support the conclusion reached in the former papers of the writer that only the cation of the salt influences the gelatin.

5. The experiments also support the suggestion made in the writer's former papers that the neutral salts act upon proteins,

which, like gelatin, are stronger acids than bases, by forming metal proteinates which dissociate electrolytically into a positive metal and a negative gelatin ion. The metal gelatinates with univalent cation (Li, Na, K, NH_4) are highly ionizable, while those with bivalent cation (Mg, Ca, Sr, Ba) are less or not at all ionizable. The anion of the salt used behaves as if it did not combine with the gelatin at all or as if it entered into a non- or less dissociable bondage.

6. These conclusions are supported by experiments with alkalies showing that if we treat powdered gelatin for half an hour with different concentrations of NaOH or KOH, and then wash the excess of alkali away, a 1 per cent gelatin solution in distilled water made from powdered gelatin thus treated is no longer precipitable with 95 per cent alcohol when the concentration of the alkali used was $\leqq$ M/128. When, however, $Ba(OH)_2$ or $Ca(OH)_2$ is used the gelatin solution in distilled water remains precipitable by alcohol no matter what concentration of the alkali was used. In this latter case, Ca and Ba gelatinate are formed which according to our assumption are not or little dissociable electrolytically; in the former case, Na and K gelatinate are formed which are highly ionizable. The fact that the alkalies give the same results as the neutral salts confirms our view that the salts combine with the gelatin, forming metal gelatinates, since the combination of alkalies with gelatin can be proved by titration experiments.

7. If we treat powdered gelatin for half an hour with different concentrations of strong monobasic acids, HNO_3, HCl, trichloroacetic acid, and then wash the excess of acid away, a 1 per cent solution of such gelatin in distilled water becomes (under the conditions of our experiment) non-precipitable with alcohol when the concentration of the acid used was $\leqq$ M/256. When we use dibasic acids, the gelatin should remain precipitable by alcohol if the excess of acid is washed away. This is approximately true for H_2SO_4 but is not true for the organic dibasic acids, such as tartaric, malic, and oxalic. A possible explanation of this discrepancy between theory and fact is offered.

8. Weak acid (acetic) and weak base (NH_4OH) act alike inasmuch as neither renders the gelatin non-precipitable (after the acid or base is washed away) which was to be expected if the effect of electrolytes on gelatin is due to a chemical reaction between the two bodies.

THE ORIGIN OF THE CONCEPTION OF PHYSIOLOGICALLY BALANCED SALT SOLUTIONS.

By JACQUES LOEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, April 24, 1918.)

An article published recently in this *Journal* contains the following sentence:

"The recent conceptions of 'physiologically balanced' solutions in their relation to biological phenomena, conceptions enlarged by Loeb and others, complicate the problem considerably by forcing a consideration of the possible antagonistic actions of different ions....."[1]

This statement is not entirely correct. Herbst had shown that if any of the constituents of the sea water was omitted the larvæ of the sea urchin could no longer develop. From this fact he drew the conclusion that each constituent of the sea water was needed for the development of the larvæ. The writer showed by his experiments on the eggs of *Fundulus*, which are laid and which develop in sea water, that this conclusion was not correct, since: (1) The eggs died very rapidly in a pure NaCl solution of the concentration in which this salt occurs in the sea water, while they could live indefinitely if a small amount of Ca was added to the NaCl; and (2) The eggs developed normally in distilled water, proving that neither Na nor Ca was needed for the development of the eggs.

These experiments, as well as others on the eggs of sea urchins and on jellyfish, led the writer in a paper published 18 years ago to the following conclusion:

"It seems to me that my experiments necessitate the introduction of a new conception, namely, that of *physiologically balanced salt solutions*. By this I mean salt solutions which contain such ions and in such propor-

[1] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1918, xxxiv, 133.

tions as to completely annihilate the poisonous effects which each constituent would have if it were alone in solution. Sea-water and blood (and approximately a mixture of 96 cc. 5/8 N NaCl + 2 cc. 10/8 N $CaCl_2$ + 2 cc. 5/8 N KCl) are physiologically balanced salt solutions.

"It will be necessary to investigate how far the conclusions of pharmacologists, botanists, and bacteriologists concerning the effects of various salts require a correction on the basis of these new facts and conceptions. Their consideration might even prove of use in problems of immunity and adaptation."[2]

As far as the writer is aware, the term as well as the conception of "physiologically balanced salt solutions" originated with this statement. In fact there was no reason to suspect the necessity of such a conception until it was shown that marine organisms, of the type of *Fundulus*, whose eggs can live and develop normally in distilled water, are nevertheless sensitive to changes in the relative proportion of the constituents of the sea water.

[2] Loeb, J., *Am. J. Physiol.*, 1899–1900, iii, 445.

A METHOD FOR THE COLORIMETRIC DETERMINATION OF LACTOSE IN MILK.

By AUGUST J. P. PACINI and DOROTHY WRIGHT RUSSELL.

(From the Pathological Laboratories of the American Telephone and Telegraph Company, New York.)

(Received for publication, April 24, 1918.)

When an aqueous solution of picric acid containing glucose is heated with an alkali, there results a Burgundy red color the intensity of which is dependent upon the quantity of sugar in solution. On this reaction is based the Lewis-Benedict[1] technique for the determination of blood sugar.

The following experimental data indicate that lactose likewise produces a color with picric acid and alkali, but also that the color is proportional to the amount of lactose present.

TABLE I.

Lactose solution. Mg. per 1,000 cc.	Colorimetric finding.	Error.	
	mg.	*mg.*	*per cent*
10	9.6	−0.4	−4.00
20	21.2	+1.2	+6.00
30	29.8	−0.2	−0.70
40	38.6	−1.4	−3.20
50	49.2	−0.8	−1.60
100	99.1	−0.9	−0.90
250	251.0	+1.0	+0.40
500	499.8	−0.2	−0.04
1,000	1,000.3	+0.3	+0.03

Between wide limits of concentration, the color formed by lactose is a linear function of the quantity in solution. This property can be utilized as a means for the colorimetric estimation of lactose in milk.

With blood it is necessary to remove the proteins previous to

[1] Lewis, R. C., and Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 61.

estimating the sugar. This is accomplished by saturating the blood with solid picric acid. The same expedient serves to remove the proteins from milk. No added precaution is required to remove the fat, since this is removed with the proteins.

Following is the method to be pursued when determining the amount of sugar in breast milk. Cow's milk is subjected to the same technique, but owing to the less quantity of sugar in cow's milk a slightly weaker standard of lactose than the one described should be used.

Technique.

Standard.—Dissolve in a saturated aqueous solution of picric acid 0.1 per cent of pure recrystallized lactose. Each cc. of standard, therefore, is equivalent to 1 mg. of lactose.

Preparation of Milk.—Dilute the human milk to be tested 1 to 100. When the supply is limited, 0.1 cc. diluted to 10 cc. will give accurate results. To about 10 cc. of the diluted milk placed in a Purdy centrifuge tube, add about 0.5 gm. of solid picric acid, and dissolve by stirring and shaking. Permit the solution to rest for about 5 minutes, then centrifuge at the usual rate (1,500 revolutions) for 10 minutes. The result is a clear yellow liquid between a sediment of precipitated proteins and a thin layer of fat. Filter through a small filter paper to remove the fat. Into a long test-tube pipette 1 cc. of the milk solution. Into a separate tube introduce 1 cc. of the standard. To each tube, add 1 cc. of a saturated sodium carbonate solution. Mix and immerse the tubes in boiling water for 20 minutes. Dilute, when cooled, both the standard and the test solution to 10 cc. Compare in the Duboscq colorimeter.

Calculation of Results.—To find the amount of lactose in the milk the following formula may be used:

$$x = \frac{S}{R} \times \frac{m}{W} \times 0.1$$

where x is the percentage of lactose in the milk, S the reading of the standard, R the reading of the unknown, m the mg. of lactose to which the standard corresponds, and W is the number of cc. of milk used. As a rule, S is set at 10, m is 1, and W is 0.01.

The following table indicates the accuracy of the method. The results show the percentage of lactose determined colorimetrically as above and by titration with Benedict's solution using the Welker and Marsh[2] method for removing the protein by means of dialyzed iron.

Specimen.	Lactose.		Difference.
	Titration.	Colorimetric finding.	
	per cent	*per cent*	*per cent*
1	7.35	7.21	−0.14
2	6.92	7.02	+0.10
3	8.04	8.00	−0.04
4	7.12	7.00	−0.12
5	7.62	7.58	−0.04
6	8.00	8.12	+0.12
7	7.10	7.18	+0.08
8	6.53	6.70	+0.17
9	5.80	5.93	+0.13
10	6.75	6.80	+0.05

A picramic acid standard may also be used.

SUMMARY.

A rapid colorimetric method based on the blood sugar method of Lewis and Benedict, is described for the determination of lactose in milk. The method is expeditious and accurate and lends itself admirably for routine laboratory procedure.

[2] Welker, W. H., and Marsh, H. L., *J. Am. Chem. Soc.*, 1913, xxxv, 823.

A NOTE ON THE ANALYSIS AND COMPOSITION OF THE SEED OF THE SILVER MAPLE (ACER SACCHARINUM).

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(From the Chemical Laboratory, New York Agricultural Experiment Station, Geneva.)

(Received for publication, April 22, 1918.)

The food shortage in various European countries has necessitated not only careful conservation of available food supplies but also has compelled the utilization of many plant constituents not ordinarily classed as foodstuffs. A similar condition is impending in this country. In order to conserve our stores of food products the exercise of the greatest economy will be necessary. In this connection it might be pointed out that numerous plant products not previously considered as food might be used to replace, in part at least, many of the more expensive and essential articles of food and thus aid in conservation.

The object of this paper is to call attention to one such substance possessing high food value which has not been utilized heretofore. We refer to the seed of the silver maple (*Acer saccharinum*). So far as we are aware this product has not been either used or analyzed previously; at least there is no record in the chemical literature of any earlier analysis. We have, therefore, undertaken an investigation of the constituents of this seed.

Silver maple is thickly planted in many parts of the country for shade and ornamental purposes. The trees are prolific producers of seeds which mature and fall early in the summer; about the middle of June in Western New York. The seeds can be easily harvested by raking them off the ground because they are usually carried only a short distance by the wind.

The seed or samara consists of two principal parts; (1) the winged pericarp, and (2), the seed surrounded by a brown seed coat or testa. The seed, after removal of the pericarp and testa,

consists of two green-colored cotyledons, separated by a thin white membrane. When dry, the wing or pericarp is brittle and can be easily separated from the seed. The testa is a very thin, brown-colored, brittle membrane which can be removed from the seed by scrubbing.

The investigation is incomplete. We have been unable to make any digestibility experiments and are therefore unable to state the nutritional value of the globulin. An organic phosphorus compound has been isolated. This substance appears to be very similar to if not identical with phytin. A globulin has also been separated in pure form but it has not been analyzed. Certain other soluble constituents require further study. The completion of the investigation has been indefinitely postponed by the war. It seems advisable, however, to publish this preliminary analysis at this time and to call attention to the food value of maple seed.

EXPERIMENTAL.

The ripe samaras were picked by hand, allowed to dry in the air for several days, and then dried in a current of air at a temperature of 40 or 50° until of constant weight. The seeds were then separated from the wings or pericarp. The loss in weight on drying was 55.34 per cent. Of the dry samara, the seed or cotyledons represented 70.00 per cent and the wings 30.00 per cent.

The outer brown seed coat was removed by rubbing the seeds together in a cloth bag and sifting through a coarse sieve. The percentage of the seed coat or testa could not be determined because the plumule and other small particles of the cotyledons broke off in the process of rubbing and became mixed with the testa.

The cotyledons, separated and prepared as described above, were powdered and analyzed. They had the following composition, calculated to the water-free basis.

	per cent
Starch	41.94
Protein (N × 6.25)	27.50
Sucrose	15.78
Pentosans	4.07
Galactan	1.08

Crude fiber	2.36
Crude fat	3.55
Ash	5.01

The following ash constituents were determined, calculated to the water-free cotyledons.

	per cent
Phosphorus	0.72
Sulfur	0.16
Chlorine	0.07
Calcium	0.09
Manganese	0.01
Magnesium	0.18
Potassium	0.70
Sodium	0.07

The dry wings or pericarp were powdered and analyzed and the following constituents determined:

	per cent
Dextrose (reducing sugar)	6.11
Sucrose	0.99
Galactan	3.45
Pentosans	15.24
Starch	14.73
Crude fiber	34.50
Crude fat	2.40
Protein (N × 6.25)	8.15
Moisture	6.29
Ash	3.98
Manganese	0.018
Sulfur	0.10
Phosphorus	0.19
Potassium	0.46
Sodium	0.08
Calcium	0.40
Magnesium	0.10

The soluble constituents of the seed were separated as follows:

1. The powdered seed was extracted with ether which removed practically all of the green color in addition to the fat. A thick green-colored oil was obtained on evaporating the ether. This oil contained a volatile substance which possessed an extremely pungent odor. It is our intention to isolate and study this substance as soon as possible.

2. After the ether had evaporated, the seed residue was extracted with 70 per cent alcohol. This extract contained from 16 to 17 per cent of solids. It contained no weighable quantity of invert sugar but after inversion it strongly reduced Fehling's solution. Estimated by this method, the sugar

was equal to 15.78 per cent of sucrose. In order to confirm the presence of sucrose in the extract, the sugar was isolated in pure form and twice recrystallized from alcohol and water. Judging by its properties and specific rotation, this substance was undoubtedly pure sucrose.

3. After extracting with 70 per cent alcohol, the seed residue was dried in vacuum over sulfuric acid. It was then extracted with 5 per cent sodium chloride solution to which sufficient barium hydroxide had been added to maintain a neutral reaction. The dissolved globulin was precipitated by ammonium sulfate. It was freed as much as possible from the mother liquor by the centrifuge, dissolved by adding a little water, filtered, and the solution dialyzed. The globulin which separated was suspended in water and dissolved by adding a little ammonium sulfate, the solution filtered, and again precipitated with ammonium sulfate to $\frac{6}{10}$ saturation. It was then centrifugalized, the precipitate dissolved in water, filtered, and again dialyzed. After filtering, dehydrating with alcohol, and drying over sulfuric acid in vacuum the substance was obtained as a nearly white heavy powder. The globulin showed no crystalline structure. Under the microscope it appeared to consist of small uniform globules. This globulin will be more fully studied and described in a subsequent publication.

4. The residue, after extracting with the dilute salt solution, was extracted with 2 per cent hydrochloric acid for the purpose of removing the soluble phosphorus compounds. The organic phosphorus compound in the extract was purified by repeated alternate precipitation, with barium hydroxide and alcohol, from dilute hydrochloric acid solution. It was finally obtained as a pure white barium salt. Lack of time has prevented the identification of this substance. In reaction and appearance, however, it seems to be very similar to the barium salt of inosite hexaphosphoric acid or phytin. We hope to study this substance more fully and will report upon it in a later publication.

Distribution of the Phosphorus.

The total phosphorus, as determined in the ash and by the Neumann method, was the same. The total soluble and the inorganic phosphorus were determined in 2 per cent hydrochloric acid extracts of the powdered seed. The time of extraction was 3 hours. The organic phosphorus is the difference between the inorganic and the total soluble phosphorus.

The relations between these forms are as follows:

	per cent
Total phosphorus	0.72
" soluble phosphorus	0.65
Inorganic phosphorus	0.15
Organic "	0.50

It will be noticed that practically all of the phosphorus is soluble in 2 per cent hydrochloric acid and also that nearly all is present in organic combination.

Distribution of the Nitrogen.

	per cent
Total nitrogen	4.40
Nitrogen soluble in 70 per cent alcohol	0.39
" " " 5 " " sodium chloride	2.06
" in residue	1.93

The small quantity of nitrogen soluble in 70 per cent alcohol is apparently non-protein in character because on evaporating the alcohol the residue is readily soluble in water.

The principal protein in the maple seed is the above mentioned globulin. In the pure state it is insoluble in water but owing to the presence of soluble salts in the seed, it is just as readily extracted by distilled water as by dilute sodium chloride.

The insoluble nitrogen remaining in the seed residue probably represents a part of the globulin which had not been dissolved by the dilute salt solution. The seed had been ground only to a No. 60 powder which was hardly fine enough to permit complete extraction of the globulin.

SUMMARY.

Attention is called to the high food value of the seed of the silver maple. The analysis shows that the chief constituents are starch, protein, and sucrose. The principal protein is a globulin. The ash contains much potassium and phosphorus. In the seed, the phosphorus is present chiefly in organic combination.

THE UREA CONTENT OF PLACENTAS FROM NORMAL AND TOXEMIC PREGNANCIES

By FREDERICK S. HAMMETT.

(From the Department of Anatomy of Harvard Medical School, Boston.)

(Received for publication, March 28, 1918.)

INTRODUCTION.

Through the impetus of new and accurate methods of blood analysis a revival of interest in the questions of fetal nutrition and excretion has come about, and Slemons and Morriss (1) and Morse (2), whose attention has been mainly directed to the problem of placental exchange, have demonstrated significant and valuable relationships between maternal blood and that of the new-born.

It is hardly in keeping with our knowledge of tissue functions in general to suppose that the placenta rests snugly and quietly on the uterine wall and observes the passage to and fro of the food and excretory products without actively influencing the exchange. Moreover its very histological structure points to the possibility of an active participation in the preparatory processes. Hence it seems logical to suppose that a biochemical study of the properties of the placental tissue would serve to assist in a better understanding of the mechanism of intrauterine development.

A study of the two most prominent end-products of protein metabolism offers opportunity for comparisons and future investigations, but since the ammonia content of placenta has been shown to increase markedly during relatively short periods (3), and the quantity of this substance in any given sample of placenta is difficult if not impossible of interpretation, this report is limited to a discussion of the urea content of placentas, for this substance has as yet given no evidence of such lability (4, 5).

It has been suggested many times that the seat of the toxemias of pregnancy lies in a perversion of placental function, but as yet no published proof exists that this is so.

The blood analyses of Losee and Van Slyke (6) fail to show any significant acidosis accompanying the toxemias of pregnancy. The determination by Farr and Williams (7), and Slemons and Morriss (1), of the combined ammonia-urea fraction of the non-protein nitrogen of the blood from toxemic cases, seems to indicate a tendency for such figures to be higher than those ordinarily obtained from normal pregnancies. Although this has given an inkling of the condition existing in such cases, the lumping

together of the nitrogen from the ammonia as such and that derived from the urea may have introduced an error, for Folin (8) has shown that even if the amounts of ammonia in freshly drawn blood are small, there is a rapid increase in this substance after a few minutes standing. The results presented in the tables in this paper speak for themselves as to the necessity for simultaneous ammonia and urea determinations in tissues if true urea values are to be obtained.

Method.

The analytical procedure followed is essentially that of Sumner (4) but is given here in detail because of some variations introduced by the author.

The placenta as soon as expelled was placed in a large glass jar with clamp cover and was either placed on ice till morning or brought to the laboratory for immediate analysis. On removal from the jar the placenta was rinsed free from adhering clots under the tap, separated from the membranes, and pulped in a meat chopper. After thorough mixing a sample was taken and put into a glass-stoppered weighing bottle, from which portions of from 5 to 8 gm. were weighed by difference into large test-tubes 20 × 200 mm. containing 2 cc. of a phosphate buffer solution. A long glass spatula was used as a means of transference, and adhering bits of pulp were washed down with a fine stream of distilled water. In this manner four samples were taken from each placenta. To two of the four tubes were added 2 cc. of the urease preparation of the Jack bean used in Folin's laboratories. Each of the four tubes was then shaken with a gentle interrupted, rotary motion thus insuring thorough mixing of the pulp with the suspending liquids. The sides of the tubes were then rinsed down with a few cc. of distilled water, the total resultant volume being between 15 and 20 cc. All were allowed to stand for half an hour. At the end of this time 8 to 10 drops of a mixture of equal parts of amyl alcohol, toluene, and ethyl alcohol, and 5 gm. of solid potassium carbonate were added to each test-tube, connection for aeration and collection of the liberated ammonia being made in the individual case immediately on the addition of the carbonate. The set was then aerated for half an hour, slowly for the first 3 minutes, and then at the rate of 5 liters of air or more per minute for the remainder of half an hour. The liberated ammonia was caught in 100 cc. long-necked flasks containing 35 cc. of distilled water and 5 cc. of 0.02 N HCl. After the half hour aeration the contents of the flask were titrated with 0.01 N NaOH, using methyl red as the indicator. The burette readings were made to 0.01 cc. Controls were regularly run as to the ammonia content of the reagents used. The results reported are calculated to mg. of ammonia or urea nitrogen per 100 gm. of tissue. The weighing of the tissues was carried on with the greatest possible speed consistent with accuracy to 0.01 gm.

TABLE I.

Data concerning the Urea and Ammonia Content of Placentas from Normal Pregnancies.

No.	Series.	NH_2N (A) per 100 gm. placenta.	Urea N (B) per 100 gm. placenta.	A + B	$\frac{A}{A+B}$
		mg.	*mg.*	*mg.*	*per cent*
1	3	4.9	2.0	6.9	71
2	4	3.4	3.9	7.3	47
3	5	1.8	4.6	6.4	28
4	6	2.3	5.2	7.5	31
5	8	3.0	1.7	4.7	64
6	9	3.5	3.6	7.1	49
7	10	2.0	3.5	5.5	36
8	11	3.9	6.7	10.6	37
9	12	2.5	5.3	7.8	32
10	13	3.7	4.4	8.1	46
11	14	3.2	4.3	7.5	43
12	15	1.8	5.7	7.5	24
13	18	1.8	4.2	6.0	30
14	19	1.4	2.7	4.1	34
15	20	1.0	3.2	4.2	24
16	21	1.2	4.9	6.1	20
17	22	1.0	2.2	3.2	31
18	23	1.6	7.5	9.1	18
19	24	1.7	4.8	6.5	26
20	25	1.0	5.8	6.8	15
21	28	2.3	8.4	10.7	21
22	34	3.3	3.8	7.1	46
23	35	1.6	5.3	6.9	23
24	36	2.6	8.3	10.9	24
25	37	2.4	6.9	9.3	26
26	39	2.0	4.7	6.7	30
27	40	0.5	9.0	9.5	6
28	41	0.8	8.3	9.1	9
29	42	0.9	6.6	7.5	12
30	47	1.5	11.1	12.6	12
31	48	0.9	7.4	8.3	11
32	49	1.2	9.2	10.4	12
33	50	1.2	8.5	9.7	12
34	51	1.9	7.6	9.5	20
35	52	2.3	12.9	15.2	15
Average		2.1	5.8	7.9	27

TABLE II.

Data concerning the Ammonia and Urea Content of Placentas from Toxemic Pregnancies.

No.	Series.	NH_3 N (A) per 100 gm. placenta.	Urea N (B) per 100 gm. placenta.	A + B	$\frac{A}{A+B}$
		mg.	*mg.*	*per cent*	*per cent*
1	7	2.6	12.4	15.0	17
2	17	1.7	10.4	12.1	14
3	26	1.6	4.4	6.0	27
4	27	2.1	16.2	18.3	12
5	29	2.1	5.6	7.7	27
6	30	0.9	4.9	5.8	16
7	31	2.1	2.5	4.6	46
8	32a	3.2	14.7	17.9	18
9	32b	3.3	15.0	18.3	18
10	33	1.6	5.3	6.9	23
11	34	4.3	9.0	13.3	32
12	43	1.6	4.7	6.3	25
13	44	4.9	8.2	13.1	37
14	45	1.5	7.2	8.7	17
15	46	3.4	24.4	27.8	12
16	53	3.4	10.2	13.6	25
17	54	2.8	7.6	10.4	27
18	55	1.4	15.0	16.4	9
19	56	5.4	10.4	15.8	34
20	57	4.0	18.8	22.8	17
Average		2.7	10.3	13.0	22

RESULTS.

Table I gives the mg. of the ammonia nitrogen and of the urea nitrogen found in 100 gm. of placental tissue from normal pregnancies. In addition there is given from the same cases the total nitrogen present as ammonia and urea and the per cent of the combined ammonia-urea nitrogen present as ammonia nitrogen. Table II shows figures for the same obtained from analyses of placentas from toxemic pregnancies.

In connection with this work certain evidence has accumulated and is shortly to be published, leading to the belief that there is more than one type of pregnancy toxemia. In view of this and in view of the fact that a classification of the toxemias of pregnancy made on the basis of clinical and other observations fails to find

correspondence in these individual results, I have not considered it worth while to attempt a separation of the results according to the present arbitrary lines of differentiation.

It is evident that the per cent of nitrogen present as ammonia in the combined ammonia-urea figures is extremely variable, ranging from 6 to 71 per cent in the normal placentas and from 9 to 46 per cent in those obtained from toxemic cases, thus indicating that for this tissue at least a separate determination of the ammonia nitrogen is necessary if accurate figures for the urea nitrogen are to be obtained.

In the normal placentas the urea nitrogen ranges from 1.7 to 12.9 mg. of urea nitrogen per 100 gm. of placenta with an average of 5.8 mg., while the range in placentas from toxic cases is higher; *viz.*, 2.5 to 24.4 mg. and the average of 10.3 mg. of urea nitrogen per 100 gm. of tissue. Moreover 77 per cent of the normal placentas have a urea nitrogen content of less than 8.1 mg. per 100 gm. of tissue, whereas but 40 per cent of the placentas from the toxemic pregnancies fall below this mark. That is to say, only 23 per cent of the normal placentas have a urea nitrogen content higher than 8.1 mg. per 100 gm., while 60 per cent of the toxemic placentas contain greater amount, and in fact 50 per cent of them have over 10.1 mg. of urea nitrogen in this amount of tissue. It is indisputable that the placentas from women suffering from a toxemia of pregnancy are generally higher in urea content than are the placentas from women whose course of gestation has been normal.

DISCUSSION AND CONCLUSION.

Two apparently valid objections present themselves in a critical review of the foregoing data. Since practically all of the cases of toxemia had received eliminative treatment for varying periods preliminary to delivery, the quantity of urea found in the placentas may not represent the actual amount accompanying this condition. Admitting this as a possibility the higher figures found in the tissue even after the eliminative treatment may be still more significant as demonstrating an increased urea as the accompaniment of the toxemias of pregnancy. The second objection, based on the great vascularity of the placenta and its

high blood content as giving results no different from what might be obtained from the blood itself, and supported by the observations of Gryns (9) and Marshall and Davis (10) on the diffusibility and distribution of urea in blood and tissues, can only be met by the opinion that the preliminary washing of the placenta free from blood would also wash out the urea. Now if the concentration of urea in the blood and tissues is approximately uniform as would appear from the results of Marshall and Davis the higher urea content of the placentas from the toxemic cases would indicate a corresponding general increased urea concentration in these conditions.

It is plain, therefore, that the toxemias of pregnancy are accompanied by a severe disturbance of nitrogen metabolism, the exact foci of which are yet to be discovered, and which results in an increased urea content of the placenta and in all probability of the body as a whole.

The material used as a basis for this report was obtained from The Boston Lying-In Hospital through the courtesy of its superintendent, Miss C. Dana, and the cooperation of the head nurses, Miss R. E. Harrington and Miss E. C. Wynne.

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NUTRITIVE FACTORS IN PLANT TISSUES.

I. THE PROTEIN FACTOR IN THE SEEDS OF CEREALS.*

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The present international crisis in the food situation has directed attention more than ever before to the comparative value of the cereals which enter commonly into the ration of man and many of the domestic animals. It is true that practical agriculture has long been concerned with the problem of the relative efficiency of the different cereal crops in connection with animal production and dairy husbandry; but the sudden necessity of a more extensive substitution of other cereal grains for wheat, particularly in the dietary of man, has given added importance to the problems at issue.

The comparison of the cereals, which resemble each other closely in being composed in major part of starch and other carbohydrates and are somewhat alike in respect to the presence or absence of different inorganic nutrients and vitamines, rests in a large measure upon the relative equivalence of their unlike proteins for the uses of nutrition. An ideal comparison, from this standpoint, requires a diet adequate and equivalent in respect to all other essentials, both organic and inorganic, known to constitute a perfect food when suitable protein is supplied therewith. It is important that all proteins of the edible parts of the cereal grains should be represented if the nutritive values of milling products produced from them are to be understood. Further-

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

more, as we have elsewhere pointed out, no comparison of this sort is convincing unless the variables of the experimental method are reduced to a minimum.[1] More precisely, it will be necessary to plan the experiments so that the animals will "receive the same amount of food during the same period of time and make the same gain in weight."[2]

In the case of the unaltered cereal grains such a program is in most cases scarcely feasible because their relatively low concentration of protein will not permit the preparation of a food mixture sufficiently rich in protein to promote growth at a normal rate. For example, the whole wheat grain contains, on an average, about 2 per cent of nitrogen. The necessary addition of certain inorganic salts, notably of calcium, and of a source of fat-soluble vitamine, such as butter fat, still further lowers the nitrogen, *i.e.* protein, content of the resulting mixture. For purposes of comparison 2 per cent of nitrogen may be regarded as equivalent at the most to 12.5 per cent of protein (N × 6.25), since this factor is certainly too high for all of the common cereals except corn. Experience in feeding rats on the fat-rich artificially prepared food mixtures which we have heretofore used has shown that a protein concentration of less than 12.5 per cent rarely suffices for normal growth.

In such experiments as have just been referred to, the diet contained at least 25 to 30 per cent of fat, which markedly enriched it in energy value. In our experience the number of grams of food eaten by growing rats is very largely determined by the calorific value of the diet. Thus much larger quantities of food mixtures containing not more than 5 per cent of fat are needed for normal growth than is the case with foods whose energy value is decidedly increased by a content of 25 per cent or more of fat. The resulting differences in food intake may amount to as much as 50 per cent by weight of the fat-rich food ingested, although the calory intake may be the same. Obviously the protein intake in the case of a fat-poor diet may thus be largely increased in comparison with the quantity of protein consumed on a fat-rich diet, solely because the *amount* of food consumed is so much

[1] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1916, xxvi, 1.

[2] Osborne and Mendel, *J. Biol. Chem.*, 1916, xxvi, 9.

larger. Unless the quantity of protein actually eaten is known, no adequate comparison between the different proteins can be made.

If no account of the food eaten is taken the outcome of the experiments may be wholly misleading. Some recent preliminary experiments of our own appeared at first sight to indicate a marked superiority of barley proteins compared with those of wheat or rice; but when gains of weight per gm. of protein eaten were calculated this superiority entirely disappeared. It is thus plain that experiments which have hitherto purported to show the relative nutritive value of different food products in respect to any one of their constituents have no comparative value unless the amount eaten is known.[3]

McCollum and his coworkers, in a series of interesting papers on the nutritive value of some of the cereal grains, such as the wheat, maize, oat, and rice, which they have published during the last 3 years, have shown that these various seeds *as a whole* contain an abundant supply of the water-soluble vitamine (water-soluble B), but that their content of fat-soluble vitamine (fat-soluble A) and their inorganic constituents are not adequate for normal growth. Regarding the value of the protein which these seeds contain, the evidence is less convincing, because no statement is made from which the amount of protein consumed can be learned. We have expressed our views on this point above as well as in earlier papers. Furthermore, the method of feeding these grains is not described; hence it is impossible to know whether the food eaten by their rats actually consisted of a mixture representing the entire grains. We have observed that when rats are supplied with entire grains they eat the embryo more eagerly than the endosperm. Since the proteins of the

[3] Thus McCollum in discussing some of our previously published data respecting the minimum proportion of some proteins needed for maintenance has stated that he has secured equally good maintenance on percentages of these proteins which were about 50 per cent lower than ours. McCollum does not state the amount of food which his experimental rats ate, but if we assume that they ate quantities of the type of food which he employed in amounts approximately equal to those eaten by rats in our experiments on foods of corresponding calorific value, the difference between the results of his experiments and ours disappears.

embryos of wheat or maize kernels are very different from those of the endosperm, both in amino-acid make-up, as well as in nutritive value, unless the method of feeding is such that no selection of the different parts of the seed is possible no final conclusions can be drawn.

A concrete illustration of the effect of a widely unlike calorific value in the food as produced by large inequalities in its content of fat is furnished by two series of our experiments in which the intakes during 5 weeks were as follows:

Rat.	Initial weight.	Final weight.	Gain.	Food composition.		Food intake.	
				Fat.	Protein.	Total.	Protein.
	gm.	*gm.*	*gm.*	*per cent*	*per cent*	*gm.*	*gm.*
4585 ♂	76	142	66	5	10.5	405	42.5
4576 ♂	72	144	72	25	15.8	269	42.5
2465 ♂	77	144	67	25	14.7	262	37.0
4589 ♀	71	121	50	5	10.5	363	38.2
4035 ♀	70	115	45	24	18.0	207	37.2
1638 ♀	73	131	64	28	17.4	242	42.1

It will be noted that despite the lower protein concentration (10.5 per cent) in some of the foods, their lower calory value per gm. induced a much larger intake with corresponding approximate equality in actual protein consumption.

Inasmuch as no properly planned experiments have been made from which the relative value of the *total protein* of any of the widely used cereals can be learned, we undertook the experiments described in the following pages. Although the results are far from satisfactory we think that under existing circumstances they should be published; for they plainly indicate how limited is our present knowledge of this subject. They may serve also to prevent the formation of hasty conclusions based on evidence already in the literature, which have been set forth in such a manner as easily to mislead those who are not in a position to give these data the critical consideration which is necessary before use can be made of them in practice.

In order to prepare foods containing the *total protein* of the different cereals we planned to remove starch by the action of diastase with the least possible loss of nitrogen. In other words, we attempted to recover all of the nitrogen of these seeds in the form of protein concentrates. It was, however, found impossible by the method first employed to obtain products containing much over 85 per cent of their total nitrogen from the entire seeds of most of the cereals. The method employed in making these protein concentrates was as follows.

About 3 kilos of the finely ground seed were mixed with 15 liters of water, and heated in a water-jacketed kettle until the starch was thoroughly gelatinized. After cooling to 55–60° about 300 cc. of a diastase preparation[4] were added and the mixture was digested until the reaction with iodine practically disappeared. It was then heated to boiling, filtered hot on large folded filters, and the residue washed with hot water and dried in a current of warm air. The failure to recover a larger proportion of the nitrogen was chiefly due to the presence of soluble nitrogenous substances which could not be separated by boiling the solution after converting the starch with diastase. To overcome this difficulty we attempted to remove all of the water-soluble constituents of the grains by extracting them thoroughly with water and then treating the residues containing the starch with diastase in the way above described. The water extracts were evaporated to dryness and the residues added to the residues remaining after converting the starch with diastase. In the case of wheat, rye, and barley the preliminary extraction was made with hot 60 per cent alcohol in order to remove the alcohol-soluble proteins because it was found that these proteins were sufficiently soluble in water to entail considerable losses of protein during the digestion with diastase, but were too little soluble in water to be removed by any practicable extraction therewith.

In this way protein concentrates were obtained which contained upwards of 90 per cent of the nitrogen of the seed; but a new difficulty was caused by the fact that some of the cereals contained so much water-soluble non-nitrogenous constituents

[4] We are indebted to Mr. Macey F. Deming of the Cereo Co. for a most liberal supply of Cereo, an active preparation of diastase.

that when these were combined with the residues remaining after removing the starch, the proportion of protein in the mixture was too small to permit its satisfactory use. Unless these protein concentrates could be fed with enough fat to make a paste which the rats do not scatter it is difficult to determine the food intake, or to compare the results of feeding with those obtained in our previous investigations. Thus the residues from whole oats and whole barley contained only about 18 per cent of protein, from corn about 25 per cent, but from wheat and rye over 30 per cent; only the two latter being rich enough in protein for making satisfactory food mixtures.

Since the labor involved in the process of preparing a sufficient supply of material by this method was so great we have made preliminary feeding experiments with the less satisfactory products containing about 80 per cent of the total nitrogen, and we expect to check the results by later trials made with better materials and under better conditions which have been indicated by the experience thus gained.

The results of feeding the products obtained in this manner have their shortcomings in several respects. In the first place the protein concentrates from *corn* used in this preliminary work contained all of the pericarp, embryo, and endosperm; those from *polished rice* contained only products derived from the endosperm; whereas the *barley* and *oat* concentrates contained the entire seed together with the glumes or husks.

When the concentrate from *polished rice* was the sole source of protein in the diet, with the exception of what was supplied by the presence of 2 per cent of dried brewer's yeast which furnished the indispensable water-soluble vitamine, rats grew well. The adequacy of the rice proteins would thus be demonstrated were it not for the supplementing effect attributable to the added yeast, which contributed about 1.2 per cent of protein to the diet, whereas the rice contributed 17.2 per cent. McCollum and Davis[5] have reported that rats will grow on diets in which polished rice is supplemented with considerable proportions of rice polishings. It seems highly probable, therefore, that the proteins of the

[5] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 181.

entire (husked) rice grain including the pericarp and embryo, *i.e.* "brown rice," are adequate for nutrition in growth.

In a study of the proteins of rice[6] it was noted that rice "does not show the marked differences which the other cereal proteins show in respect to their amino-acid make-up when compared with the majority of food proteins, and particularly with most tissue proteins."

McCollum and Simmonds later stated that: "Rice proteins, like those of maize and wheat are of relatively low biological value."[7] In our extensive experience with protein concentrates from the maize endosperm we have never succeeded in obtaining growth in any way comparable with that represented by the experiments with rice, shown in the chart, unless they were supplemented with a much larger proportion of adequate protein. As has already been pointed out, however, satisfactory comparisons between the proteins of the entire seeds cannot be made until preparations of these protein concentrates richer in protein and containing more nearly all of the nitrogen of the seed can be used. The unfavorable impression of the rice proteins gained by McCollum and his associates may be due to the fact that the method of feeding employed by them did not permit a sufficiently high intake of these proteins to produce as much growth as might have occurred had a larger amount of protein been eaten.

It is interesting to note that more than 10 years ago Hopkins[8] wrote: "This matter of qualitative differences in proteins may be of no small significance in dietaries. It may account for what I believe is proved by experience—that rice may serve the races which rely upon it as an almost exclusive source of protein, while wheat is only suitable for races that take a much more varied dietary."

Our experience with the *barley* concentrates shows that the proteins from this source are adequate to promote growth when they are furnished in sufficient abundance. In these experiments

[6] Osborne, T. B., Van Slyke, D. D., Leavenworth, C. S., and Vinograd, M., *J. Biol. Chem.*, 1915, xxii, 276.

[7] McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1917, xxxii, 361.

[8] Hopkins, F. G., *Analyst*, 1907, xxxi, 395.

(see chart) yeast was added during part of the trials before it was realized that these barley residues were sufficiently rich in water-soluble vitamine to make the yeast additions unnecessary. Growth still continued after removal of the yeast from the food.

Regarding the nutritive value of the proteins of the *oat* kernel little is known. Commercial protein concentrates from this seed are not produced.

According to McCollum, Simmonds, and Pitz: "The oat kernel seems to contain proteins of a poorer quality than either the maize or wheat kernel."[9] They further state: "Casein does not appear to supplement the proteins of the oat kernel in a very satisfactory manner. The addition of 10 per cent of casein to 9 per cent of oat proteins, the other dietary factors being properly adjusted, does not induce growth at the maximum rate as do similar combinations of casein with wheat proteins or with maize proteins. Combinations of gelatin and oat proteins in about equal proportions have proven in our experience uniformly vastly superior to the similar casein and oat protein combinations." Again they state: "There can be no doubt that gelatin furnishes an amino-acid mixture which makes good the deficiencies of the oat proteins in a much greater degree than does casein."

Why this should be true is not easily understood from our present knowledge of the amino-acid make-up of these protein products. The addition of 10 per cent of casein to any diet, as was done in the experiments just referred to, in itself furnishes adequate protein to promote considerable growth provided a sufficient absolute quantity is ingested; so that one might well inquire whether, in such a case, the proteins of the oat kernel offered any supplementing factor to the casein, rather than *vice versa*. In our own experience we have found that when one-quarter of the oat protein was replaced by either casein or gelatin (the total percentage of protein remaining constant) the rate of growth was markedly increased, out of all proportion to the slight rise in the food intake. This supplementing effect of the gelatin and casein is shown in the following table where it will be noted that the food intakes in the second period were not much greater than in the first, despite the fact that the rats were considerably larger and might therefore be expected to require correspondingly increased quantities of food.

[9] McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1917, xxix. 342, 343.

Gains in Body Weight during 2 Weeks.

Rat.	Food.*	Body weight. Initial.	Body weight. Final.	Gain in weight.	Food intake.	Food eaten per gm. gain.
		gm.	gm.	gm.	gm.	gm.
4377 ♂	Oat concentrate = 2.7 per cent N.	69	79	10	92	9.2
	Oat concentrate = 2.03 " " "					
	Gelatin = 0.69 " " "	79	102	23	87	3.8
4378 ♀	Oat concentrate = 2.7 " " "	67	73	6	79	13.2
	Oat concentrate = 2.03 " " "					
	Casein = 0.64 " " "	73	102	29	96	3.3
4379 ♂	Oat concentrate = 2.7 " " "	65	74	9	91	10.1
	Oat concentrate = 2.03 " " "					
	Casein = 0.64 " " "	74	103	29	92	3.2

* Composition of the food mixtures:

	per cent	per cent	per cent
Oat protein concentrate	45	33.75	33.75
Casein			4.50
Gelatin		4.50	
Salt mixture†	5	5.00	5.00
Starch	19	25.75	25.75
Brewer's yeast, dried	2	2.00	2.00
Butter fat	18	18.00	18.00
Lard	11	11.00	11.00

† The composition of the salt mixture is given in *J. Biol. Chem.*, 1917, xxxii, 317.

In the two periods here shown for each of the rats the calorific values of the foods were alike except that in the first period the food contained a somewhat larger proportion of the oat concentrate, and consequently a little more indigestible carbohydrate. These experiments differ from those of McCollum and coworkers in having a small amount of yeast, equivalent to not more than one-fourteenth of the total nitrogen of the food, added to the diet. The content of yeast was also constant throughout, so that the increased gain in weight in the second period must have been due to the effect of the casein or gelatin. That the gelatin and casein actually supplemented the amino-acid deficiencies of

the oat proteins is shown by the fact that not even maintenance is possible when gelatin furnishes all of the protein in the diet even when it is fed in large quantities; and when casein is fed alone in the concentration in which it was given in this food, only very slow growth is promoted.

McCollum believes that gelatin supplements oat proteins better than casein; but our experience indicates that the reverse may be true, as the following table shows.

Gains in Body Weight in 4 Weeks.

Rat.	Food.*	Body weight.		Gain in weight.	Total food intake.	Food eaten per gm. gain.
		Initial.	Final.			
		gm.	gm.	gm.	gm.	gm.
4377 ♂	Oat concentrate = 2.03 per cent N. Gelatin = 0.69 " " "	79	122	43	192	4.47
4378 ♀	Oat concentrate = 2.03 " " " Casein = 0.64 " " "	73	122	49	206	4.20
4379 ♂	Oat concentrate = 2.03 " " " Casein = 0.64 " " "	74	144	7	231	3.30

* Composition of the food mixtures:

	per cent	per cent
Oat protein concentrate	33.75	33.75
Casein		4.50
Gelatin	4.50	
Salt mixture†	5.00	5.00
Starch	25.75	25.75
Brewer's yeast, dried	2.00	2.00
Butter fat	18.00	18.00
Lard	11.00	11.00

† The composition of the salt mixture is given in *J. Biol. Chem.*, 1917, xxxii, 317.

Here it will be noted that the more economical gains were made by the rats on the casein mixture. Since it was not until the ratio between the food eaten and weight gained was calculated that this superiority was demonstrated, this experiment offers a further illustration of how erroneous may be the conclusions as to

the relative values of different foods without a knowledge of the amount of food and protein eaten.

Although the preliminary results reported in this paper do not permit strict comparisons of the nutritive value of the kernels of these cereals, nevertheless they offer presumptive evidence that the *total proteins of rice and barley*, in contrast with maize and oats, when furnished in diets containing 16 to 17 per cent of protein, *supply enough of all of the amino-acids essential for growth*.

We have never yet been able to feed a sufficient quantity of the proteins of the *maize* concentrate to effect normal growth, although we have evidence that all of the essential amino-acids are present in some part of the seed. For example, we have been able to promote normal growth when maize glutelin furnished practically all of the protein in an otherwise adequate food mixture. Rats have made considerable growth on a diet containing protein furnished wholly by corn embryo; likewise with a mixture of corn gluten (*i.e.* corn endosperm proteins) and corn embryo when a sufficient quantity of the latter was present.[10] McCollum, Simmonds, and Pitz[11] state:

"We have attempted several times to nourish young rats with a diet restricted to the germ of the maize kernel, and to mixtures of the maize germ and whole ground maize in several proportions. These attempts have been uniformly unsuccessful. Two samples of germ from different dealers were employed. We are not certain just what treatment these had received, but both appeared to be wholesome products. It is evident from these trials that it is not easy, if at all possible, to make up a satisfactory ration wholly derived from the corn kernel and its parts."

This does not necessarily imply that the *proteins* of the maize embryo are inferior. With the embryo separated mechanically in the preparation of hominy considerable growth was attained. A sample of distiller's oil cake, which is prepared from the maize germ, likewise furnished protein efficient in promoting growth. We are not prepared to state whether long continued use of corn germs in large quantity will exhibit the toxic properties that have been attributed to the germs from wheat.[12]

[10] Osborne and Mendel, *J. Biol. Chem.*, 1917, xxix, 69.

[11] McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1916–17, xxviii, 155.

[12] Cf. Hart, E. B., Steenbock, H., and Humphrey, G. C., *Univ. Wisconsin Agric. Exp. Station, Bull. 287*, 1918.

Our experiments indicate that it ought to be possible to make an animal grow on a diet in which the maize kernel is the sole source of protein, provided a preparation of the total proteins could be obtained which would permit feeding them in sufficient quantity so that enough of those amino-acids which are present in certain of the proteins and not in the others would be available to meet the minimum nutritive requirements of the organism.

Since other experiments[13] have shown that yeast is adequate for growth when fed as the sole source of protein, it is possible that the 2 per cent of yeast, which was incorporated in the foods containing cereal protein concentrates to insure the presence of an abundance of the water-soluble vitamine, was a more efficient supplement for the cereal proteins than might be anticipated. This is indicated by the fact that in the case of the foods containing the rice, corn, and oat concentrates the rats receiving yeast grew somewhat better than did those receiving protein-free milk as a source of water-soluble vitamine, although the total amount of additional nitrogen furnished by 2 per cent of yeast was practically the same as that supplied by 28 per cent of the protein-free milk; *i.e.*, 0.19 per cent of the food. That the yeast proteins probably did not supplement the barley proteins is shown by the fact that the rats continued to grow at a normal rate on the barley diet after the yeast was removed from the food, thus proving that at least some of the water-soluble vitamine present in the whole seed was left in the protein concentrate prepared by the action of diastase.

The oat residues were likewise found to contain some of the water-soluble vitamine, because rats were able to grow when the yeast was omitted from the ration, provided gelatin was present to supplement the oat proteins. When both the gelatin and yeast were removed, the rats declined, indicating that the initial growth on the oat-yeast diet was due to the supplementing value of the yeast proteins.

We reiterate that the observations here recorded must be looked upon as preliminary in character and preparatory to further studies now in progress. It may be helpful to other investigators, however, to have some of the difficulties that we have encountered pointed out.

[13] Unpublished data.

CHART.

CHART I. Showing the growth of rats on diets in which the protein was furnished in the form of concentrates from various cereal kernels: barley (B), rice (R), and oats (O).

The composition of the diets containing *barley* concentrates was as follows:

	Rats 4410, 4405, 4400, 4389, 4396, 4413.	
	Period 1.	Period 2.
	per cent	*per cent*
Barley concentrate	58–65	65–67
Salt mixture*	4	4
Brewer's yeast, dried	2	
Starch	7–0	2–0
Butter fat	18	9
Lard	11	20

* The composition of the salt mixture is given in *J. Biol. Chem.*, 1917, xxxii, 317.

In Period 2 for this group the yeast was omitted from the diet without apparent detriment to growth.

The composition of the diets containing the *rice* concentrates was as follows:

	Rats 4418, 4384, 4397.		Rats 4395, 4422, 4383.	
	Period 1.	Period 2.	Period 1.	Period 2.
	per cent	*per cent*	*per cent*	*per cent*
Rice concentrate	24–28	28	24 28	28
"Protein-free milk"				
Salt mixture*	4	4		4
Brewer's yeast, dried	2	0·1 gm. per day.		0·1 gm. per day
Starch	41–37	39	19	39
Butter fat	9–18	9	9–18	9
Lard	20–11	20	20–11	20

When either the yeast or the "protein-free milk" was removed from the diets in a period subsequent to those shown in the chart, the lack of the water-soluble vitamine was manifested by a prompt decline in the weight.

Rats 4379, 4377, and 4378 were kept on diets containing *oat* concentrates as indicated in the text (p. 529). The failure to grow adequately on the oat diet and the supplementary value of both casein (C) and gelatin (G) are apparent.

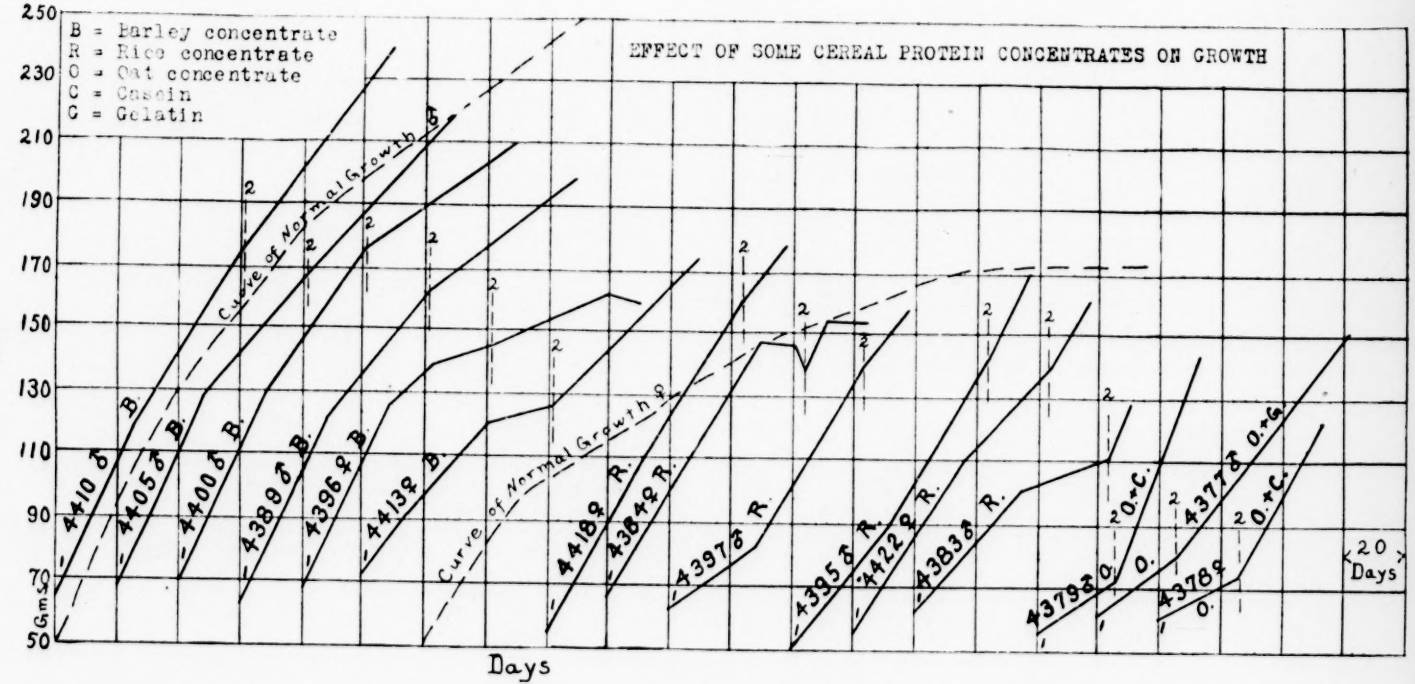
EFFECT OF SOME CEREAL PROTEIN CONCENTRATES ON GROWTH
B = Barley concentrate
R = Rice concentrate
O = Oat concentrate
C = Casein
G = Gelatin
Curve of Normal Growth ♂
Curve of Normal Growth ♀
Grams
Days
20 Days
4410 ♂ B.
4405 ♂ B.
4400 ♂ B.
4388 ♂ B.
4396 ♀ B.
4413 ♀ B.
4418 ♀ R.
4384 ♀ R.
4397 ♂ R.
4395 ♂ R.
4422 ♀ R.
4383 ♂ R.
4379 ♂ O.
O.
4378 ♀ O. + C.
4377 ♂ O. + G.
O. + C.
O.

CHART I.

MILK AS A SOURCE OF WATER-SOLUBLE VITAMINE.*

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In our early feeding experiments with isolated food substances the failure either to induce substantial growth in young rats or to satisfy completely the maintenance requirement of older animals during long periods on the mixtures then tested led us to study the value of milk in the diet.[1] Having secured very favorable results with a food paste consisting of dried milk powder, starch, and lard, we proceeded to prepare what was termed "protein-free milk"[2] which enabled us to undertake a large number of trials of different proteins for their nutritive significance. The use of about 28 per cent of protein-free milk as the sole source of inorganic salts and what may now be termed water-soluble vitamine in the food mixtures—a procedure which was successful in many hundreds of experiments on rats—was based on the favorable results previously obtained with the food pastes containing the equivalent proportion of milk, in the form of 60 per cent of the dried milk powder. We continued to use this quantity because experience had shown that "addition of not inconsiderable portions (5 to

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

[1] Osborne, T. B., and Mendel, L. B., Feeding Experiments with Isolated Food-Substances, *Carnegie Institution of Washington, Publication 156*, pts. i and ii, 1911.

[2] Osborne and Mendel, *Carnegie Institution of Washington, Publication 156*, pt. ii, 1911, 80. Attention is here called to an error in the description of the preparation of protein-free milk on p. 81, line 1, in which 1.64 cc. should read 164 cc.

30 per cent) of the actual milk food to the earlier inefficient protein mixtures is incapable of bringing about growth in any degree equal to that at once initiated when the protein-free milk is added in relative abundance."[3]

In attempting to study the rôle of lipoids in nutrition Stepp[4] had found that milk restored the nutritive efficiency of diets which, by thorough extraction with alcohol and ether, had been rendered inadequate for the nutrition of mice. A little later Hopkins[5] published his noteworthy observations on the effect of additions of fresh milk in securing growth in rats which otherwise failed to grow upon so called "synthetic" dietaries consisting of mixtures of purified proteins, fats, carbohydrates, and salts. From present day standards it is assumed that the addenda of milk used by the different investigators promoted nutrition and growth by furnishing accessory factors or vitamines rather than any of the long familiar nutritive dietary units. Hopkins' experiments differ from our own particularly in respect to the astonishingly small amount of milk that sufficed to render adequate an otherwise ineffective food mixture. This is even more striking in view of the fact that most of the milk solids consist of substances which fall into the categories of the common foodstuffs. Thus Hopkins says:

"In my experiments, while the artificial diet consisted of casein, fat, starch, sugar and inorganic salts, the addendum consisted of milk itself; but this was given in such small quantity that the total solids contained in it amounted to no more than from 1 to 3 or 4 per cent of the whole food eaten. This small addition induced normal growth upon dietaries which without it were incapable even of maintenance. A special feature of my experiments was the rigorous use of controls. In each and every experiment two sets of rats, chosen carefully so as to show correspondence in the weight, sex, and origin of the individuals contained in them, were fed side by side. The sole difference in treatment consisted in the administration of the minute ration of milk to one of the sets compared. In some experiments after the relative rates of growth had been compared for a week or two, the small milk ration was transferred to the set which had been previously fed without it. In all cases the influence of the milk upon growth

[3] Osborne and Mendel, *Carnegie Institution of Washington, Publication 156*, pt. ii, 1911, 83.

[4] Stepp, W., *Z. Biol.*, 1912, lvii, 135.

[5] Hopkins, F. G., *J. Physiol.*, 1912, xliv, 425.

was so large that it could not have been due to any alteration in the quality of the protein eaten or in its ratio, nor, in my own belief, to the presence of any known milk constituent."

The quantities of milk just referred to could yield at most an equivalent of 0.3 gm. of protein-free milk per day, thereby constituting no more than 5.2 per cent of the food mixture, in contrast with the 28 per cent which we have been accustomed to use when this product has served as the source of vitamine.

In a subsequent communication Hopkins and Neville[6] again describe the nutritive failure of rats on synthetic diets—a result which we can substantiate—and their improvement as the result of small additions of milk. Thus they state:

"To six of the above set of rats, after the decline in their weight had begun, 2 cc. of milk *per diem* were given. An immediate betterment of the general condition of the animals followed; growth was reestablished and the health then maintained. In another experiment six rats were put upon Osborne and Mendel's diet, but were given milk from the first. In each case the animal grew."

Hopkins and Neville further state, however:

"When the small ration of milk was given each day in advance of feeding with the Osborne and Mendel diet the food consumption remained of the same order, and did not rise to the amount consumed by the rats in Hopkins' earlier experiments. The resulting growth though quite definite and steady was distinctly slower than in the experiments mentioned."

We were long ago impressed by the apparent discrepancies in the quantitative relations of the amounts of milk required to furnish the vitamine factor in our experiments in contrast with those of Hopkins. This was especially true in view of our repeated failures to obtain equally satisfactory results when small proportions of protein-free milk were employed. For example, even with 14 per cent in the diet the rats failed sooner or later; but with very few exceptions they responded promptly when 28 per cent was given. With quantities varying from 0.28 per cent to 7 per cent the rats failed to complete their growth although some of them made very satisfactory gains during the first few weeks on such diets. These experiments indicate that individual animals vary greatly in their requirements for this water-soluble

[6] Hopkins, F. G., and Neville, A., *Biochem. J.*, 1913, vii, 97.

vitamine. We have noted the same variability with respect to the quantities of yeast which must be fed to different animals to induce similar amounts of growth.

28 per cent of protein-free milk in the food mixture supplies the equivalent of 30 to 50 cc. of whole milk per day for rats eating from 6 to 10 gm. of food per day when the composition and calorific value of the food approximate that employed by both Hopkins and us. Hopkins himself has commented upon the apparent discrepancies between our earlier results and his. A careful study of his charts will show that the animals which received 5 cc. of milk made better gains than those receiving smaller quantities, and particularly so when the less purified "protene"—a commercial casein product from milk—was employed in his food mixtures. Furthermore the growth of Hopkins' rats even with the larger milk additions was, as a rule, less rapid than is true of rats on our successful food mixtures. Without some information regarding the rate of normal growth of the animals in Hopkins' colony it would be unfair to assume that his experimental animals were far behind the average. In any event the contrast between his experiments with and without milk is sufficiently striking to overshadow the secondary consideration as to whether the quantities of milk employed by him permitted the *maximum* growth which we have taken as our standard.

Newer experiments in which dried *whole* milk was used by us as the source of water-soluble vitamines have indicated that less than 24 per cent is unsatisfactory for inducing rapid growth in food mixtures of the following general type:

	per cent
Casein	18
Milk powder	24
Salt mixture	3
Starch	28
Butter fat	9
Lard	18

This mixture contains the equivalent of 10 per cent of protein-free milk. McCollum and Davis[7] have reported experiments in which smaller proportions of *skimmed milk* powder added to diets containing polished rice induced growth in animals. From a

[7] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 181.

quantitative standpoint the results are somewhat conflicting; and the records are not directly comparable with those of Hopkins or ourselves owing to the absence of data regarding food intakes. Presumably, however, even under the most favorable conditions in McCollum and Davis' experiments the milk equivalent of the *skimmed milk* powder fed by them was of decidedly larger magnitude than in the case of Hopkins' experiments.

In searching for a tenable explanation of the (quantitatively) inferior results obtained by us we have thought of the possibility that the manipulations, and particularly the heating, incident to the preparation of the dried protein-free milk as well as the milk powder, might produce a deterioration or loss of the vitamine factor. This seemed somewhat plausible in view of the alleged destruction of the antiscorbutic properties of milk through pasteurization. Accordingly we attempted to duplicate the experience of Hopkins by feeding diets consisting of casein or edestin, starch, a salt mixture, lard, and butter fat, along with which fresh milk was offered in varying quantities to the animal. When 2 cc. of milk per day were given the animals were rarely able to make more than very slight gains in weight, and many of them were barely maintained. Inasmuch as most of the market milk locally available is pasteurized prior to delivery we took the added precaution of securing unpasteurized milk from a high class dairy farm. This did not alter the outcome of our experiments; indeed we have no reason to believe that the nutrition-promoting properties of milk are lost by brief periods of heating.

Comparative trials made with approximately equivalent amounts of protein-free milk as such, and of fresh milk not incorporated with the food mixture, have shown substantially similar outcomes (see Charts I and II). Not until at least 16 cc. of fresh milk per day were supplied along with the food mixture, was anything approaching a normal rate of growth secured. Even this amount sometimes failed. We were at first inclined to attribute such failure to the considerable volume of fluid intake which this addendum entailed, so that the additional quantities of the basal ration sufficient to permit rapid growth were not consumed by the animals. This hypothesis was soon negatived, however, by the demonstration that the further addition of a small amount of brewer's yeast (about 25 mg.) per day served

to facilitate growth by markedly raising the food intake in addition to the continued ingestion of the fluid milk. 16 cc. of milk per day had not therefore limited the rats' ability to *eat* sufficient food for growth.

That the deficiency of diets containing the lesser amounts of milk involves the vitamine factor is rendered more than probable by the fact that these comparatively small additions of yeast, the highly efficient growth-promoting power of which we have discussed elsewhere,[8] sufficed to render the previously inadequate ration satisfactory for growth. As a rule the most significant outcome of the yeast additions (which were fed apart from the rest of the food and therefore could not have altered its flavor) was a larger food intake. We are not prepared to assert, however, that an augmented food intake is the sole effect of the incorporation of these vitamine-containing products in the dietary.

We are at a loss to explain the apparent differences, in respect to the efficiency of milk as an accessory or vitamine factor in the diet, in the experiments of Hopkins and ourselves. It scarcely seems plausible that milk from different sources, even though produced by cows on unlike feeds, should account for the wide variations noted; in fact we have used samples from several local sources, always with the same result. However, it may be recalled that according to McCollum, Simmonds, and Pitz,[9] the efficiency of milk to promote the growth of the young of the same species is believed to be dependent upon a suitable intake of vitamines with the diet, thus suggesting that they are not produced *de novo* in the body of the lactating animal.

> It is stated that the vitamines "pass into the milk only as they are present in the diet of the mother, and that milks may vary in their growth-promoting power when the diets of the lactating animals differ widely in their satisfactoriness for the growth of young."[9]

Protein factors cannot be involved here, inasmuch as we have shown that even on a diet inadequate with respect to its protein, the milk of the mother may be suitable for the growth of her young.[10]

[8] Osborne and Mendel, *J. Biol. Chem.*, 1917, xxxi, 149.

[9] McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916, xxvii, 33.

[10] Osborne and Mendel, *J. Biol. Chem.*, 1912, xii, 473.

The evidence of various investigators regarding the antineuritic value of milk, however, is not at variance with our experience in the study of growth. Cooper[11] found milk far inferior to various animal tissues in curing or preventing avian polyneuritis. According to Gibson and Concepción[12] in feeding experiments with fowls, pigs, and dogs, in the Philippines, the results obtained showed the antineuritic vitamines to be present in milk in slight amounts only, and that the continued feeding of either fresh or autoclaved milk without suitable additions to the diet induced symptoms of beri-beri. The authors conclude that the antineuritic powers of milk are so slight that in infant feeding the diet should be extended as soon as possible; and that the young of healthy mothers are probably born with a reserve supply of the so called vitamine substances sufficient to maintain them in good nutritive condition until the time when they begin to eat other foodstuffs.

From a practical standpoint a more precise understanding of the quantitative relations of the vitamines is highly desirable. The fact that such diverse cellular structures as yeast, wheat embryo, corn germs, and glandular tissues[13] show a surprising richness in growth-promoting properties aside from their protein and mineral content, is of immediate significance at a time when milk is still so uniquely valued for its nutrient virtues. Of course, the problem of the fat-soluble vitamine in milk is not here considered. It is not unlikely, however, that the need of children and other growing animals for the water-soluble vitamine, beyond the earlier stages of development when milk admittedly satisfies the nutritive requirements, may not be adequately filled by some of the current or enforced dietary practices. Thus with a too scanty allowance of milk, a liberal inclusion of products from cereals rendered poor in vitamine by milling, of sugar, fats, and few additional animal products other than meat (which has been shown to contain relatively little of the water-soluble vitamine[14]), it is not surprising if disasters sometimes manifest themselves.

In view of the results of Hopkins' experiments it has become

[11] Cooper, E. A., *J. Hyg.*, 1912, xii, 436; 1914, xiv, 12.

[12] Gibson, R. B., and Concepción, I., *Philippine J. Sc., B*, 1916, xi, 119.

[13] Osborne and Mendel, *J. Biol. Chem.*, 1917, xxxii, 309; 1918 xxxiv, 17.

[14] Osborne and Mendel, *J. Biol. Chem.*, 1917, xxxii, 369.

generally believed that milk is one of the richest sources of the water-soluble vitamines among our food products. Whatever explanation may ultimately be found for the much larger amounts of milk needed to promote normal growth in our rats compared with Hopkins' rats, it seems advisable for the present in practice to use a liberal amount of milk when this is depended on to supply any considerable proportion of this most necessary food factor.

A particular case of this kind is that of infant feeding where it is customary to reinforce the supply of calories by diluting top milk and adding milk sugar. Under these circumstances the child is supplied with a food that contains a relatively smaller proportion of the water-soluble vitamine than does the original cow's milk. While milk thus modified may contain sufficient vitamine as long as the food intake is normal, if for any reason the child's appetite fails the vitamine supply is reduced and endless dietary troubles may easily result. It is not improbable that a large part of the difficulties of artificially feeding babies is due to this cause, and that these can be obviated as successfully by securing an adequate supply of this indispensable constituent of a suitable diet as has been the case in feeding animals on artificial diets since we have learned how properly to provide this food factor.

This is a practical question which deserves prompt investigation, for the best way to reinforce the vitamine content of the diluted milk is not yet evident, chiefly owing to our still limited knowledge of the distribution of this vitamine in nature. Such investigations as have been made demonstrate that the proportion of water-soluble vitamine in the various vegetable and animal tissues differs greatly, and it is probable that sooner or later some way will be found to a solution of the problem of successful infant feeding.

CHARTS.

CHART I. The records in Series A show the comparative effect of additions of varying quantities—2 to 16 cc.—of fresh cow's milk to a diet otherwise devoid of water-soluble vitamine and consisting of

	per cent
Casein	18.0
Salt mixture*	4.5
Starch	50.5
Butter fat	9.0
Lard	18.0

It will be noted that continued vigorous growth was not attained until the larger quantities of milk were supplied. When addition of 16 cc. of milk failed to promote growth on the above diet a small amount of yeast (25 mg. of brewer's yeast, dried) often sufficed to improve nutrition, and the effect of its removal was promptly manifested (see Rats 4438, 4443). That the milk furnished some vitamine is shown by the speedy decline after its removal from the diet, in Rat 4434, despite the presence of the yeast. Without milk at least 100 mg. of yeast per day were required to promote growth in the rats on this diet, as shown in the final periods for Series A, Rats 4434, 4438, 4443.

Series B likewise shows the slight promoting effect of 2 cc. of milk in the first periods, in contrast with the experience of Hopkins. When fresh milk was replaced by protein-free milk (in the periods indicated by the dotted lines) no better results were obtained until larger quantities, *i. e.* more than 5.6 per cent. of the food were furnished. Small supplements of yeast, as well as increments in the quantity of protein-free milk sufficed to facilitate growth.

In Series B the food consisted of

	per cent
Edestin	18.0
Salt mixture*	0.0–4.5
Protein-free milk	28.0–0.0
Starch	25.0–48.5
Butter fat	9.0
Lard	20.0

* The composition of the salt mixture is given in *J. Biol. Chem.*, 1917, xxxii, 317.

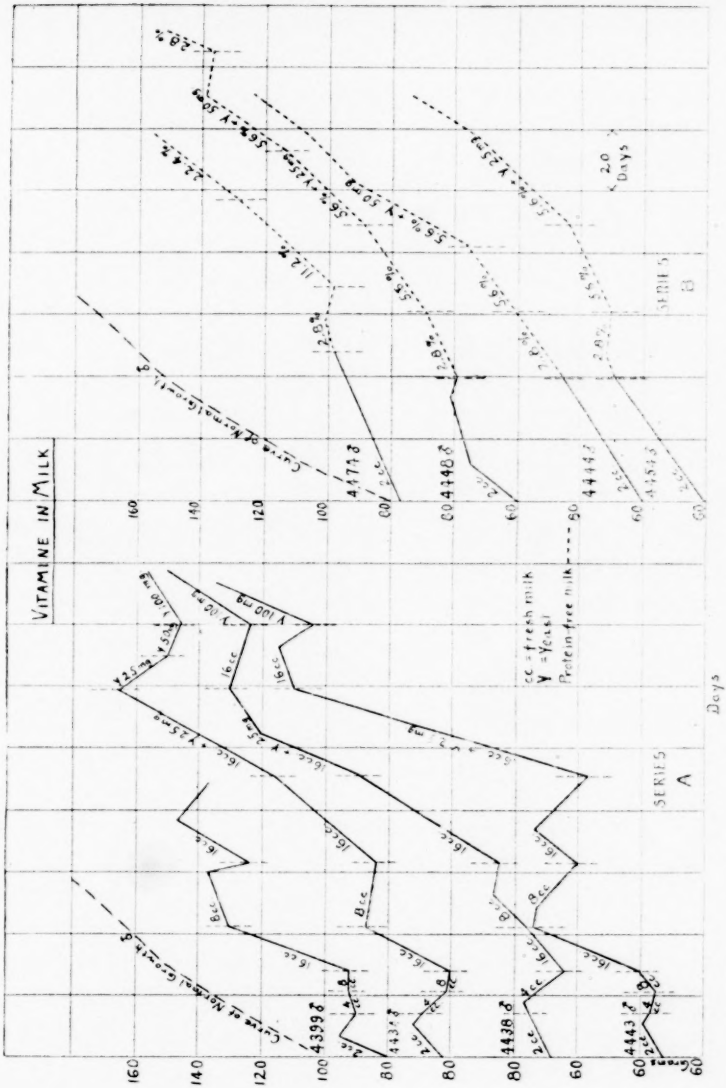
VITAMINE IN MILK
SERIES A
SERIES B
Days
2.0 Days
cc = fresh milk
Y = Yeast
Protein-free milk
Curve of Normal Growth
Curve of Normal Growth
4477 ♂
4446 ♂
4399 ♂
4438 ♂
4443 ♂
4444 ♀
4445 ♀
160
140
120
100
80
60

CHART I.

CHART II. Showing the comparative effect of supplements of fresh milk (unbroken lines) or protein-free milk (dotted lines) as sources of water-soluble vitamine added to diets consisting of

	per cent
Casein or edestin	18.0
Protein-free milk	0.0–22.4
Salt mixture*	4.5–1.0
Starch	47.7–31.6
Butter fat	9.0
Lard	18.0–20.0

It will be noted that growth was not vigorous until considerable quantities of fresh milk or protein-free milk were used. Small quantities comparable to the amounts of fresh milk used in Hopkins' experiments were of little avail. The use of yeast (Y) even in small quantities always promoted the nutritive result.

Rat 4484 which grew unusually well on a diet containing only 5.6 per cent of the protein-free milk illustrates the occasional variations which are observed whenever large numbers of nutrition experiments are made to ascertain the minimum of some essential dietary factor. Generalizations should, therefore, only be drawn from a considerable number of individual trials.

* The composition of the salt mixture is given in *J. Biol. Chem.*, 1917, xxxii. 317.

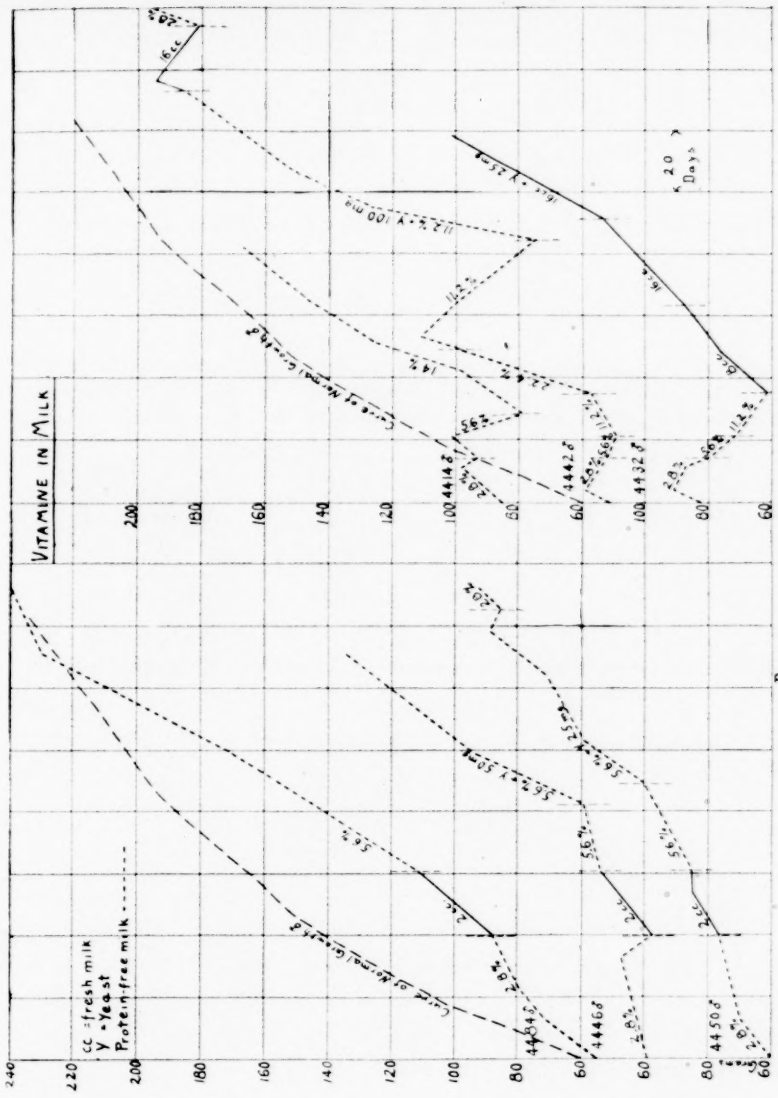
VITAMINE IN MILK
cc. fresh milk
y = Yeast
Protein-free milk
Days

CHART II.

CHART III. Showing the effect of additions of varying amounts of milk (recorded in cc.), milk powder (per cent), or yeast (Y), as sources of water-soluble vitamine in a diet consisting of

	per cent
Casein	18.0
Milk powder	0.0–48.0
Salt mixture*	4.5– 1.0
Starch	50.0–18.0
Butter fat	9.0
Lard	18.0

As in Charts I and II, growth was far from satisfactory until the milk additions were considerable in amount. Yeast invariably augmented the effect of the milk.

* The composition of the salt mixture is given in *J. Biol. Chem.*, 1917, xxxii, 317.

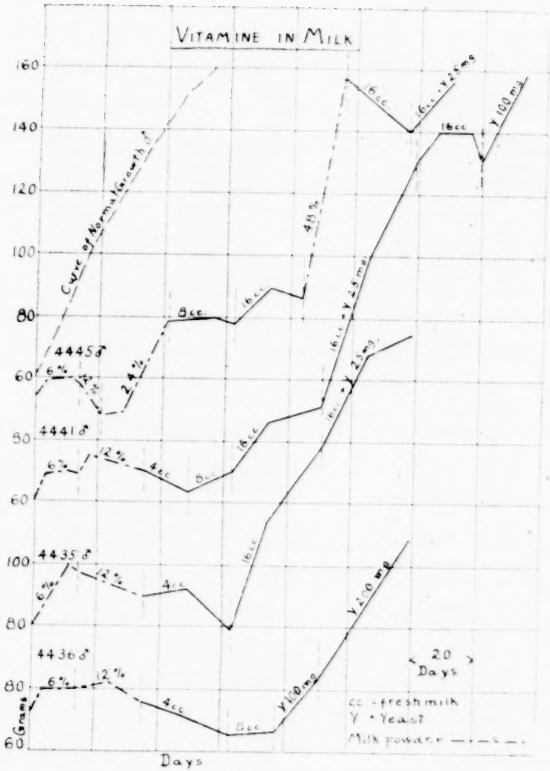
VITAMINE IN MILK
Curve of Normal Growth
4445♂
4441♂
4435♂
4436♂
6%
12%
24%
40%
8cc
16cc
4cc
Y 2.5 mg
Y 100 mg
Y 200 mg
20 Days
cc = fresh milk
Y = Yeast
Milk powder
Grams
Days

CHART III

HYDROGEN ION CONCENTRATIONS OF VARIOUS INDICATOR END-POINTS IN DILUTE SODIUM HYPOCHLORITE SOLUTIONS.*

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In the preparation of Dakin's hypochlorite solution it has been customary to adjust the reaction by the use of powdered phenolphthalein. After precipitating the calcium of the bleaching powder with Na_2CO_3, Dakin added boric acid to the strongly alkaline hypochlorite solution until it no longer colored powdered phenolphthalein. Daufresne modified this technique by using sodium bicarbonate in place of boric acid, but still used powdered phenolphthalein as the test for the reaction of the solution. Such a solution has frequently been described somewhat loosely as a "neutral" solution of hypochlorite, but it is not neutral. Because of the bleaching action of the hypochlorite, the end-points of indicators in hypochlorite solution are quite different from the end-points of the same indicators in ordinary solutions. A much greater alkalinity is required to give a color with powdered phenolphthalein in the presence of hypochlorite than in its absence. Moreover, the degree of alkalinity required in a hypochlorite solution to give a color with powdered phenolphthalein is much greater than that required to give a flash of color with alcoholic phenolphthalein. As alkalinity in these solutions increases their stability, their irritating effects upon the skin and tissues, and their solvent action on necrotic tissue (Dakin; Dakin and Dunham; Carrel and Dehelly; Fiessinger and coworkers; Taylor and Austin), it seemed desirable to define as accurately as possible the hydrogen ion concentrations actually indicated by these

* A preliminary report appeared in *Proc. Soc. Exp. Biol. and Med.*, 1917, xv, 41.

two end-points.[1] We have used powdered phenolphthalein and 1 per cent alcoholic solution of phenolphthalein. To these we have added 0.1 per cent alcoholic solution of *o*-cresolphthalein, which in hypochlorite solutions has an end-point between those of powdered and alcoholic phenolphthalein. *o*-Cresolphthalein, first made by Fraude (1880), has been studied and recommended by Clark and Lubs as a more brilliant indicator than phenolphthalein over about the same range.

The hypochlorite solutions have been prepared by passing chlorine gas through solutions, either of sodium hydroxide or of sodium carbonate, of known concentration. The chemical reactions under these conditions may be represented as follows:

With sodium hydroxide:

(1) With equivalent molecular proportions of sodium hydroxide and chlorine:

$$Cl_2 + 2\ NaOH = NaCl + NaOCl$$

(2) With excess of chlorine:

$$Cl_2 + NaOH = NaCl + HOCl$$

With sodium carbonate:

$$2\ Na_2CO_3 + Cl_2 = 2\ NaHCO_3 + NaCl + NaOCl$$

Actually at any given moment in the reaction there exist proportions of Na_2CO_3, $NaHCO_3$, NaOCl, and HOCl determined by the alkalinity and degree of chlorination. Starting with either Na_2CO_3 or NaOH, as the chlorine is introduced, the alkalinity of the solution is diminished and the total hypochlorite (NaOCl + HOCl) increased. With any given solution of either type therefore the change in the thiosulfate titration as the chlorine is introduced is an approximate gauge of the change in C_a.

EXPERIMENTAL.

A series of experiments was made in which, starting with a known solution of sodium hydroxide, chlorine gas was introduced

[1] Clowes recognized the variations that occurred in hypochlorite solutions, prepared with powdered phenolphthalein as the sole test of reaction and suggested a further control by titration of the alkali content.

to the end-point of one or more of the three indicators, and at these points samples of the solution were titrated with thiosulfate. The results are given in Table I. It is evident that there is a fairly constant relation between the original concentration of the sodium hydroxide and the thiosulfate titrations at the end-points to powdered phenolphthalein and to *o*-cresolphthalein. The end-point to *o*-cresolphthalein is a little less alkaline than that to powdered phenolphthalein. The titration at the end-point to alcoholic phenolphthalein is much less constant. This is due to two factors: first, the difficulty of reading the evanescent flash of color; second, and we believe more important, the formation at this C_H of other acids, $HClO_2$, $HClO_3$, $HClO_4$, as well as HClO,

TABLE I.

Sodium Hydroxide and Chlorine.

Original solution NaOH per 100 liters	0.1 N thiosulfate per 10 cc. at end-point to		
	Powdered phenolphthalein.	*o*-Cresolphthalein.	Alcoholic solution of phenolphthalein.
gm. mols.	*cc.*	*cc.*	*cc.*
28.3		28.5	33.1
27.8	27.0		35.3
27.8			32.8
27.7	27.3	30.1	37.2
27.7	27.4	29.4	
27.5			31.6
26.9	26.7		38.2
12.8		13.1	
12.8		12.9	

the three former being produced in varying amounts depending upon the precise conditions of the experiment and thus altering considerably the relation between the C_H and the thiosulfate titration.

Similar experiments starting with a known sodium carbonate solution (Table II) showed much greater variation in the relation between the C_H and the thiosulfate titration at all three end-points. This is presumably due to the partial escape of CO_2 liberated in the reaction. As the extent to which this escape of CO_2 takes place varies somewhat depending upon the rate at which the

chlorine is introduced, the fineness of the bubbles in which it is introduced, the thoroughness of the stirring, and the temperature of the solution, this variation between C_H and thiosulfate titration is readily understood.

In addition to the thiosulfate titrations, analyses were made in these solutions of the total chlorides, of the alkalies, and of the carbonate contents, so that the approximate composition of the solutions could be calculated. The ratios of $\frac{HOCl}{NaOCl}$ at the various indicator end-points as determined in the sodium hydroxide experiments were made use of in the calculation of the composition of the carbonate solutions at these same end-points. Finally, from the calculated $\frac{NaHCO_3}{Na_2CO_3}$ ratios of the carbonate solutions at these end-points the C_H values were calculated by McCoy's figures.

TABLE II.

Sodium Carbonate and Chlorine.

Original solution Na_2CO_3 per 100 liters.	0.1 N thiosulfate for 10 cc. sample at end-point to		
	Powdered phenolphthalein.	o-Cresolphthalein.	Alcoholic solution of phenolphthalein.
gm. mols.	*cc.*	*cc.*	*cc.*
28.8	17.2		35.7
28.6			34.3
27.4	12.5	27.0	
27.3	19.8		35.0
27.1	13.8	27.7	33.6
27.0	14.9		35.0
12.9	9.8	13.2	16.9
12.9	8.9	13.6	17.4

$$C_H = \frac{NaHCO_3}{Na_2CO_3} \times \frac{6.0 \times 10^{-11}}{0.8}$$

All hydrogen ion concentrations will be expressed by Sörensen's symbol pH; *i.e.*, $pH = \log \frac{1}{C_H}$.

Methods.

End-Points.—The introduction of the chlorine gas into the alkaline solutions was stopped at frequent intervals and 3 to 4 cc. portions of the hypochlorite solutions were removed and placed in small test-tubes to be tested as follows:

Powdered Phenolphthalein.—A few mg. of the powder were placed upon the solution and the tube was shaken. The first test failing to develop the red or pink color was taken as the end-point.

o-Cresolphthalein.—Several drops (8 to 10) of 0.1 per cent alcoholic solution of *o*-cresolphthalein were squirted into the solution from a medicine dropper. A definite flash of purplish red color was considered an indication of greater alkalinity than the end-point. After the end-point is reached a dirty grayish color may appear upon addition of the indicator, but this was disregarded.

Alcoholic Phenolphthalein.—A 1 per cent alcoholic solution was used in exactly the same way as the 0.1 per cent alcoholic solution of *o*-cresolphthalein. The flash of color is red, not purplish, in this case.

Analyses.—The alkali solutions before chlorination were titrated with decinormal acid. The chlorinated solutions were titrated for total hypochlorite (NaOCl and HOCl), with 0.1 N sodium thiosulfate, using 10 cc. samples to which an excess of KI and acetic acid had been added. For the determination of free NaOH or HOCl, chlorides, and CO_2 in the chlorinated solutions, 25 cc. samples were treated with 10 cc. of H_2O_2 to change NaOCl and HOCl to NaCl and HCl. When the evolution of gas was complete, 10 cc. portions were titrated for acid or alkali, and 5 cc. portions were titrated for chlorides by Volhard's method. CO_2 was estimated by the method of Van Slyke. Corrections were made for the acidity of the H_2O_2 added and for the dilution by the H_2O_2. The figures charted are thus corrected.

Experiments with Sodium Hydroxide.

The titration figures per 10 cc. and the calculated composition of the solutions of the seven experiments of this group are given in Table III.

TABLE III.

Sodium Hydroxide Solutions.

Experiment No.	Titration figures. Titration of 10 cc. 0.1 N HCl	0.1 N NaOH	0.1 N thiosulfate per 10 cc.	0.1 N NaCl per 10 cc.	Calculated composition of solutions per 100 liters. NaOH	NaOCl	NaCl	HOCl	Sodium per 100 liters.	HOCl / NaOCl
	cc.	*cc.*	*cc.*	*cc.*	*gm. mols.*	*gm. mols.*	*gm. mols.*	*gm. mols.*	*gm. atoms*	
I. (*a*) Original solution	27.40*	—	0.00	0.00	27.4				27.4	
(*b*) End-point to powdered phenolphthalein	0.45*	—	27.00	13.65	0.4	13.5	13.6		27.5	
(*c*) " " alcoholic "	—	9.95*	35.35	37.25		8.2	19.6	9.8	26.8	
II. (*a*) Original solution	26.85*	—	0.00		26.9				26.9	
(*b*) End-point to powdered phenolphthalein	0.40*	—	26.65		0.4	13.3	13.3		27.0	
(*c*) " " alcoholic "	—	11.80*	38.20			7.3	19.6	11.8	26.9	
III. (*a*) Original solution	27.80*	—	0.00	0.00	27.8				27.8	
(*b*) End-point to alcoholic phenolphthalein	—	5.75*	32.75	33.40		10.8	17.0	5.7	27.8	
IV. (*a*) Original solution	27.70†	—	0.00	0.00	27.7				27.7	
(*b*) End-point to powdered phenolphthalein	0.25†	—	27.26	27.80	0.2	13.6	14.2		28.0	
(*c*) " " *o*-cresolphthalein	—	2.54†	30.09	30.90		12.7	15.9	2.5	28.6	0.197
V. (*a*) Original solution	27.70†	—	0.00	0.00	27.7				27.7	
(*b*) End-point to powdered phenolphthalein	0.39	—	27.35	27.70	0.4	13.7	14.0		28.1	
(*c*) " " *o*-cresolphthalein	—	1.47†	29.40	29.92		13.2	15.2	1.5	28.4	0.114

VI. (*a*) Original solution	12·80†	—	0·00	0·00	12·8							
(*b*) End-point to *o*-cresolphthalein	—	0·45†	12·92	13·50		6·01	7·04	0·45	13·1			0·075
VII. (*a*) Original solution	12·80†	—	0·00	0·00	12·8							
(*b*) End-point to *o*-cresolphthalein	—	0·49†	13·12	13·70		6·07	7·14	0·49	13·2			0·081

* Indicator used, phenolphthalein.
† " " sodium alizarin sulfonate.

TABLE IV.

Sodium Carbonate Solutions.

Experiment No.	Titration figures: 0.1 N HCl per 10 cc. to Phenolphthalein	Titration figures: 0.1 N HCl per 10 cc. to Methyl orange	Titration figures: 0.1 N Hypochlorite per 10 cc.	Titration figures: 0.1 N NaCl per 10 cc.	Titration figures: CO_2 per 10 cc.	HOCl NaOCl ratios used in calculation	Calculated composition of solutions per 100 liters: NaOH	NaCO$_3$	NaHCO$_3$	NaOCl	NaCl	HOCl	Sodium per 100 liters.	Calculated hydrogen ion concentration.
	cc.	*cc.*	*cc.*	*cc.*	*cc.*		*gm. mols.*	*gm. mols.*	*gm. mols.*	*gm. mols.*	*gm. mols.*	*gm. mols.*	*gm. atoms*	*pH*
VIII. (*a*) Original solution	27.00	55.00	0.00	0.00	67.00			27.00	1.00				55.5	
(*b*) End-point to powdered phenolphthalein	14.30	39.70	14.90	15.20	62.00			14.30	11.10	7.40	7.8		54.9	10.2
IX. (*a*) Original solution	27.35	53.85	0.00	0.00	64.00		0.30	26.90					54.2	
(*b*) End-point to powdered phenolphthalein	14.70	41.90	12.50	12.96				14.70	12.50	6.20	6.70		54.3	10.2
(*c*) End-point to *o*-cresolphthalein	4.20	27.00	27.00	27.30	42.00	0.197		6.40	16.40	11.30	13.80	2.20	54.3	9.7
						0.075		5.15	17.65	12.55	13.80	0.95	54.3	9.6
X. (*a*) Original solution	27.10	55.80	0.00	0.00				27.10	1.60				55.8	
(*b*) End-point to powdered phenolphthalein	13.20	41.30	13.78	14.50				13.20	14.90	6.89	7.61		55.8	10.1
(*c*) End-point to *o*-cresolphthalein	3.89	27.00	27.73	28.80		0.197		6.16	16.95	11.60	14.93	2.27	55.8	9.7
						0.075		4.86	18.25	12.90	14.93	0.97	55.8	9.55

| | | |

The method of calculation will be given in detail for Experiment I. For convenience all concentrations are given as gram molecules per 100 liters.

Experiment I.

Original NaOH Solution.—From the titration of the alkalinity of the original solution the initial concentration of 27.4 gm. molecules per 100 liters of NaOH was calculated.

NaOH Solution Chlorinated Till It No Longer Colors Powdered Phenolphthalein.—This solution required, after decomposition of the NaOCl by H_2O_2, 0.45 cc. 0.1 N HCl per 10 cc., and this must be attributed only to free NaOH. The sodium hypochlorite was determined by titration with thiosulfate. The NaCl was taken as the difference between the total NaCl found and the NaOCl which had been converted by the H_2O_2 into NaCl and was therefore included in the Volhard estimation.

NaOH Solution Chlorinated to End-Point to Alcoholic Phenolphthalein.—This solution upon titration was acid and required 9.95 cc. of 0.1 N NaOH. The titratable acid, which it will be remembered was measured after decomposition of the NaOCl to NaCl and of the HOCl to HCl by H_2O_2, must have been due to HCl derived from HOCl and to such oxidized acids, $HClO_2$, $HClO_3$, and $HClO_4$, as may have been formed. In Experiment Ic (see Table III) the chlorides found are in excess of what would be produced in accordance with the equations given above, which call for as many molecules of chlorides as of NaOCl and HOCl combined. However, chloride figures are always somewhat higher than the total hypochlorite. Foerster and Dolch have shown that there is some liberation of oxygen from NaOCl with the formation of NaCl. This is, in part, the explanation of the excess of chlorides. In addition, at the less alkaline end-points of *o*-cresolphthalein and alcoholic phenolphthalein the formation of chloric and other oxidized acids, which is associated with the formation of NaCl, accounts for a part of this excess of chlorides. It is not possible to calculate from the measurements available in our experiments the precise amounts of these oxidized acids present, but the small excess of chloride over that calculated indicates that the quantities must be small, probably less than 0.3 gm. molecules

per 100 liters, even in those solutions which have the largest amount of HOCl. Such small quantities can be disregarded and we therefore calculate the entire acid found as HOCl. This subtracted from total hypochlorite gives the NaOCl, and the total hypochlorite subtracted from the total chloride gives the NaCl content of the solution before the addition of peroxide. As a check upon the analyses, the sodium content in gm. atoms per 100 liters has been calculated as the sum of the Na in the NaCl and NaOCl, and is given in a separate column. These figures should be theoretically constant for each experiment. From these experiments it will be seen that at the end-point of powdered phenolphthalein all of the hypochlorite is present as NaOCl and a trace of free alkali still persists. At the end-point to *o*-cresolphthalein a small fraction of hypochlorite is present as the acid so that the ratio $\frac{\text{HOCl}}{\text{NaOCl}}$ varies from 0.075 to 0.197.

Experiments with Sodium Carbonate.

In Table IV are given the results of the experiments made with sodium carbonate solutions. The titration of the alkali was done by the phenolphthalein-methyl orange method in order that the carbonate and bicarbonate might be calculated separately. In the original solutions and at the end-point to powdered phenolphthalein there are no acids other than the $NaHCO_3$. However, we know from Experiments IV to VII that in the chlorinated solutions at the end-point to *o*-cresolphthalein there is an appreciable amount of HOCl present which will have been converted by the peroxide into HCl before the phenolphthalein-methyl orange titration is carried out. Correction in the latter titration figures must therefore be made for this added HCl. This has been done on the assumption that the $\frac{\text{HOCl}}{\text{NaOCl}}$ partition in the carbonate solution, within the range of hypochlorite concentration used, is the same for a given indicator end-point as in the sodium hydroxide solutions. By taking, therefore, the extreme values obtained from this ratio in the hydroxide experiments and using each of these ratios in succession in calculating the carbonate experiments at the *o*-cresolphthalein end-point, the limits of

composition of the latter solutions can be estimated. This calculation may be illustrated from Experiment IX (Table IV). Total hypochlorite = 13.5. Using ratio $\frac{HOCl}{NaOCl} = 0.197$, then of the total hypochlorite 11.3 = NaOCl and 2.2 = HOCl. Correcting the alkali titration figures for the HOCl we obtain with phenolphthalein $\frac{1}{2}$ $Na_2CO_3 = 4.2 + 2.2 = 6.4$; with methyl orange $NaHCO_3 + Na_2CO_3 = 27 + 2.2 = 29.2$. From these we derive 6.4 Na_2CO_3 + 16.4 $NaHCO_3$.

The pH values are calculated from the $\frac{NaHCO_3}{Na_2CO_3}$ ratios by McCoy's formula. It will be seen that the pH at this end-point to powdered phenolphthalein lies between 10.2 and 10.1. The end-point to *o*-cresolphthalein lies between 9.2 and 9.7. The solution with the most alkaline end-point to this indicator had twice the hypochlorite concentration of the solution with the least alkaline. Since the shifting of the end-point with these indicators from the neutral point toward the alkaline side is the result of the oxidizing action of the hypochlorite it would be expected that the displacement of the end-point will be greater in a stronger hypochlorite solution. At the end-point to alcoholic phenolphthalein the solution is so unstable as to make the analyses too uncertain for calculation in this manner. It is probable, however, by analogy from the other two end-points and the pH values calculated for them and by consideration of the relation between the thiosulfate titration figures for the three end-points as given in Table II, that the end-point of alcoholic phenolphthalein is about as much less alkaline than that of *o*-cresolphthalein as the latter is less alkaline than that of powdered phenolphthalein; that is, for alcoholic phenolphthalein the end-point lies near a pH of 8.5 to 8.8.

The marked loss which occurs in the CO_2 from the carbonate solution during the introduction of chlorine is shown by the analyses in Experiments VIII and IX.

DISCUSSION.

It is evident from the results obtained in these experiments that in these solutions a much greater degree of alkalinity is required to produce color with powdered phenolphthalein than is

required to give a flash of color with alcoholic phenolphthalein solution. The color production in these hypochlorite solutions is probably dependent upon the relative rates of bleaching, on the one hand, and of formation of red alkaline phenolphthalate on the other. The color intensity is due to the amount of dye present in solution and to the degree of alkalinity. In using powdered phenolphthalein in the less alkaline solutions the rate of oxidation is so rapid that the minute amount of dye that dissolves in the solution is bleached before color can be detected. As the solution becomes more alkaline the intensity of color increases and also apparently the rate of oxidation decreases until a point is reached where the rate of color production is greater than the rate of the bleaching. This is at a pH of about 10.2.

When one adds a small drop of alcoholic phenolphthalein solution to a hypochlorite solution the color is discharged so rapidly that the eye cannot see it. If several drops are added rapidly—as was always done in these experiments—the amount of phenolphthalein changed to the color product is sufficient to persist for an appreciable time. The flash of color with alcoholic phenolphthalein is not therefore an exact end-point, for it may easily be shifted through a considerable range. It is also clear that sodium hypochlorite solutions such as are used in clinical work (Dakin's solution) when prepared so as to just fail to color powdered phenolphthalein are still strongly alkaline in reaction. In fact, a truly neutral solution or even one sufficiently alkaline to color alcoholic phenolphthalein would contain so large a proportion of HOCl as to be quite unstable and unsuitable for clinical use. We have found that a solution intermediate in alkalinity between the end-points of alcoholic and solid phenolphthalein gives the end-point to *o*-cresolphthalein described in this paper. This solution is still sufficiently alkaline to be stable for practical purposes. It is possible that such a solution may be tolerated upon skin and other tissue surfaces which would not bear the more alkaline solution at the end-point to powdered phenolphthalein. Unquestionably in clinical use hypochlorite solutions of widely varying reaction have in the past been used in spite of the fact that all failed to give a color with powdered phenolphthalein. It would seem desirable, at least in the more careful studies of the effects of these solutions, to define somewhat more closely the locations

of their pH. This may be done within certain limits by noting the behavior of the solution with *o*-cresolphthalein as well as with powdered phenolphthalein; in the less alkaline ranges even alcoholic phenolphthalein may be used for this purpose.

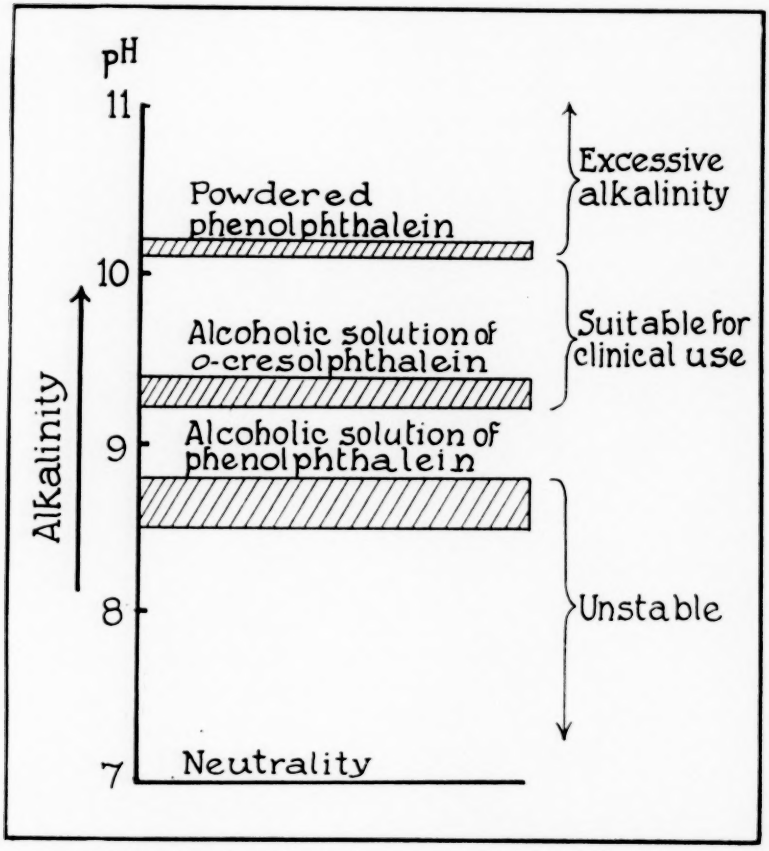

FIG. 1. Approximate hydrogen ion concentrations at end-points to powdered phenolphthalein, alcoholic solution of phenolphthalein, and alcoholic solution of *o*-cresolphthalein in 0.5 per cent sodium hypochlorite solutions.

The relation between the hydrogen ion concentration at these indicator end-points can be readily visualized by means of the accompanying chart (Fig. 1).

Application to the Preparation of Dakin's Solution.

From a consideration of the carbonate concentration employed in the above experiment it is evident that a hypochlorite solution having a pH of about 9.4 can be readily prepared by passing chlorine gas through a sodium carbonate solution of an initial concentration of 14 gm. to the liter (13.2 molecules per 100 liters) till sodium hypochlorite is formed in a concentration of 0.5 per cent as shown by titration with thiosulfate. A solution so prepared has proved most satisfactory for clinical purposes in this hospital.

SUMMARY.

1. It is shown that the end-points of indicators in dilute sodium hypochlorite solutions are different from the end-points of the same indicators in ordinary solutions.

2. A method is described for studying the hydrogen ion concentrations of dilute sodium hypochlorite solutions by means of the end-points of powdered phenolphthalein, of phenolphthalein in alcoholic solution, and of *o*-cresolphthalein in alcoholic solution.

3. The end-point to powdered phenolphthalein in a 0.5 per cent sodium hypochlorite solution is at a pH of about 10.1. All of the hypochlorite is present as the salt (NaOCl).

4. The end-point to alcoholic solution of *o*-cresolphthalein in 0.5 per cent sodium hypochlorite solution is at a pH of about 9.3. The same end-point in a 1 per cent sodium hypochlorite solution is at a pH of about 9.6. Of the total hypochlorite in these solutions from 7 to 17 per cent is present as HOCl. This less alkaline solution is sufficiently stable to be satisfactory for clinical use.

5. The end-point to alcoholic solution of phenolphthalein in similar solutions cannot be precisely estimated by our method owing to the rapid decomposition of the HOCl, which constitutes from 35 to 60 per cent of the total hypochlorite of such solutions. The pH is probably, however, about 8.5 to 8.8. This solution is highly unstable because of the low alkalinity, and is also for other reasons unsuitable for clinical use.

6. It is suggested that for certain purposes the hydrogen ion concentration of dilute hypochlorite solutions be defined more

closely than has been the custom in the past. For this purpose the end-points of the indicators established above are of value.

7. For preparing sodium hypochlorite solution (Dakin's solution) from chlorine and sodium carbonate, a method is outlined which secures the required hypochlorite concentration and also the desired alkalinity, simply by using definite amounts of carbonate and chlorine.

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NOTE ON THE CARBON DIOXIDE CONTENT OF URINE.

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In the course of our investigation of certain problems which involved the ingestion of small quantities of sodium bicarbonate, it was found convenient to use the carbon dioxide content of the urine as a measure of bicarbonate excretion. In this connection the question at once presented itself as to what variations must be looked for in the excretion of urinary carbon dioxide under ordinary normal conditions. A consultation of the literature proved unfruitful. The recorded determinations of carbon dioxide are few in number,[1–5] and have been made under conditions of such diversity that correlation of the various results appeared difficult if not impossible.

In view of this scarcity of data we have felt it desirable to place on record the results of a limited series of observations on the carbon dioxide content of normal human urine. The results of these experiments are tabulated below.

Our determinations of carbon dioxide were made by means of the apparatus recently described by Van Slyke.[6] Our figures represent therefore not only free CO_2 but also CO_2 existing in the form of bicarbonates and carbonates. It was early found that the amount of CO_2 present in any urine varies inversely though not proportionately with the hydrogen ion concentration. The amount of urine to be taken for a determination varies, therefore, from 5 cc. in strongly acid urines to 1 cc. in urines passed by persons on an

[1] Pflüger, E., *Arch. ges. Physiol.*, 1869, ii, 165.
[2] Ewald, C. A., *Arch. Anat. u. Physiol.*, 1873, 1.
[3] Wurster, C., and Schmidt, A., *Zentralbl. Physiol.*, 1887, i, 421.
[4] Van Nüys, T. C., and Lyons, R. E., *Am. Chem. J.*, 1892, xiv, 15.
[5] Lieblein, V., *Z. physiol. Chem.*, 1895, xx, 77.
[6] Van Slyke, D. D., *J. Biol. Chem.*, 1917, xxx, 347.

alkaline vegetarian diet. In the urine of persons consuming an ordinary mixed diet and excreting about 1 liter of urine per day, 2 to 3 cc. of urine will usually be found a convenient amount to use.

As urine contains considerable and varying amounts of gas other than carbon dioxide (probably air) we have in every case made a double extraction and have absorbed the carbon dioxide by means of caustic potash according to Van Slyke's directions. The determinations of the hydrogen ion concentration were made by the colorimetric method according to the technique described by Henderson and Palmer.[7]

At the beginning of the work the question at once presented itself as to whether the carbon dioxide content of urine changes on storage. It is conceivable that in alkaline urines the carbon dioxide content might increase on standing, and that in acid urines a diminution of CO_2 might take place. Experimental work however proved that this was not the case, for, as indicated by the following results, no change is demonstrable in the carbon dioxide content of either acid or alkaline urines, preserved with chloroform, and kept in well stoppered containers at room temperature for 24 hours. As in our work it was always possible to complete our analyses shortly after the closing of the 24 hour period we have not inquired into the question of the feasibility of determining carbon dioxide in urines stored for longer periods.

Urine No.	pH	CO_2 per 100 cc. urine 15 min. after voiding.	CO_2 per 100 cc. urine 24 hrs. after voiding
		cc.	cc.
1	5.0	0.70	0.69
2	5.3	2.80	2.80
3	5.7	3.80	3.80
4	6.2	12.4	12.0
5	7.0	28.0	27.7

In Table I we have collected the results obtained by the determination of total carbon dioxide in twenty-six 24 hour urines collected from six persons, in several of whom it was possible to obtain a series of urines collected when the subject was taking a variety of diets of a fairly definite character.

[7] Henderson, L. J., and Palmer, W. W., *J. Biol. Chem.*, 1912–13, xiii, 398.

TABLE I.

Carbon Dioxide in Urine.

Subject.	Day.	Volume of urine.	pH	Total CO_2 for 24 hrs.	Food.
		cc.		*cc.*	
Mr. McL.	1	990		34.7	Ordinary mixed diet.
	2	1,050		59.1	" " "
	3	940	6.0	46.1	Low protein diet containing much vegetables.
	4	930	6.3	362.0	" " "
Mr. H.	1	2,300	6.0	101.2	" " "
	2	2,700	6.1	106.2	" " "
Mr. L.	1	600	5.4	4.3	3 weeks on high protein diet.
	2	740	5.7	9.6	3 " " " " " "
Miss W.	1	900		29.7	Ordinary mixed diet.
Mr. T.	1	1,030		27.0	" " "
	2	1,430		45.2	Low protein diet containing much vegetables.
	3	2,000		256.0	" " "
	4	1,660		132.0	" " "
	5	2,080		148.0	High protein.
	6	1,480		31.6	" "
	7	1,220		22.7	" "
Miss M.	1	870	5.6	15.6	Eggs, beef, cheese, bread, butter, milk.
	2	850	5.4	11.0	" " " " " "
	3	850	5.3	9.3	" " " " " "
	4	830	5.3	9.1	" " " " " "
	5	820	5.1	16.4	Mixed diet.
	6	700	6.0	115.0	" "
	7	1,000	5.5	20.0	" "
	8	962	5.8	111.2	" "
	9	620	6.2	118.0	Low protein diet containing much vegetables.
	10	1,000	7.0	333.0	" " "

TABLE II.

Carbon Dioxide in Urine (Subject A).

Day.	Time.	Urine. Volume.	pH	Total CO_2.	Remarks.
		cc.		cc.	
1	7–10 a.m.	130	6.1	5.4	Breakfast, 6.34 a.m.: banana, bread, butter, coffee.
	10 a.m.– 1 p.m.	110	6.2	6.6	
	1–4 "	62	5.2	0.8	Lunch, 1 p.m.: roast beef, macaroni, butter, bread, jam.
	4–7 "	76	6.2	4.5	
	7–10 "	120	6.9	12.1	Dinner, 6 p.m.: roast lamb, potatoes, peas, lettuce, salad, cheese, mince pie.
	10 p.m.– 4 a.m.	200	6.3	10.8	
	4– 7 "	190	7.1	34.7	Total CO_2 for 24 hrs., 74.9 cc.
3	7–10 a.m.	205	6.1	13.5	Breakfast, 6.45 a.m.: 2 eggs, bread, butter, ½ pt. milk, coffee.
	10 a.m.– 1 p.m.	180	6.2	6.5	
	1– 4 "	93	5.6	1.35	Lunch, 1 p.m.: 6 eggs, bread, butter, egg and milk pudding.
	4– 7 "	113	6.0	4.5	
	7–10 "	135	5.2	7.0	Dinner, 6 p.m.: 200 gm. beefsteak, bread, butter, egg and milk custard.
	10 p.m.– 4 a.m.	280	6.1	9.5	
	4– 7 "	195	6.3	5.3	Total CO_2 for 24 hrs., 47.6 cc.
5	7–10 a.m.	448	5.7	15.2	Breakfast, 6.45 a.m.: 1 banana, 1 orange, 1 slice bread, butter, coffee.
	10 a.m.– 1 p.m.	99	5.3	1.4	
	1– 4 "	85	6.0	1.5	Lunch, 1 p.m.: potato 300 gm., cabbage, celery, lettuce, 1 slice bread, butter.
	4– 7 "	129	6.1	4.9	
	7–10 "	380	7.0	34.2	Dinner, 6.30 p.m.: cauliflower, potatoes, carrots, tomatoes, onions, 1 slice bread, butter.
	10 p.m.– 4 a.m.	315	7.1	31.2	
	4– 7 "	175	7.1	15.4	Total CO_2 for 24 hrs., 103.8 cc.*

TABLE III.

Carbon Dioxide in Urine (Subject B).

Day.	Time.	Urine. Volume.	pH	Total CO_2.	Remarks.
		cc.		cc.	
1	7–10 a.m.	182	7.0	75.5	Breakfast, 7.15 a.m.: 1 grape fruit, 350 gm. potato.
	10 a.m.– 1 p.m.	88	7.0	32.1	
	1– 4 "	42	6.1	5.9	Lunch, 1 p.m.: 400 gm. potato, string beans, apple, fruit salad, butter.
	4– 7 "	51	7.2	48.9	
	7–10 "	215	7.0	86.0	Dinner, 6.15 p.m.: 300 gm. potato, cauliflower, tomato, apple, 1 slice bread, butter.
	10 p.m.– 4 a.m.	180	6.9	27.7	
	4– 7 "	95	5.3	1.04	Total CO_2 for 24 hrs., 277 cc.
3	7–10 a.m.	74	5.0	0.74	Breakfast, 7.15 a.m.: 2 eggs, bread, butter, milk.
	10 a.m.– 1 p.m.	130	4.9	0.91	
	1– 4 "	81	5.0	0.43	Lunch, 1 p.m.: 4 eggs, cheese, bread, butter.
	4– 7 "	95	5.1	0.17	
	7–10 "	165	5.1	0.90	Dinner, 6.30 p.m.: 200 gm. beefsteak, bread, butter.
	10 p.m.– 1 a.m.	95	5.0	0.17	
	1– 4 "	115	4.8	1.38	
	4– 7 "	90	5.0	0.67	Total CO_2 for 24 hrs., 5.37 cc.
5	7–10 a.m.	277	6.2	11.1	Same food as on 1st day. 3,800 cc. water taken during day and night.
	10 a.m.– 1 p.m.	602	6.3	38.5	
	1– 4 "	277	6.0	8.8	
	4– 7 "	729	6.5	93.3	
	7–10 "	750	7.0	212.2	
	10 p.m.– 1 a.m.	485	7.0	90.7	
	1– 4 "	350	6.3	20.0	
	4– 7 "	310	5.8	6.0	Total CO_2 for 24 hrs., 481 cc.
7	7–10 a.m.	153	5.0	1.7	Same food as on 3rd day. 1,800 cc. water taken during day.
	10 a.m.– 1 p.m.	520	5.4	11.4	
	1– 4 "	337	5.1	1.2	
	4– 7 "	170	5.1	1.0	
	7–10 "	150	5.2	2.1	
	10 p.m.– 1 a.m.	165	5.2	Trace.	
	1– 4 "	120	5.2	1.1	
	4– 7 "	170	5.0	0.5	Total CO_2 for 24 hrs., 19.0 cc.

The results presented in this table indicate therefore the general level of the carbon dioxide content of normal urine under various dietary conditions. The minimum value 4.3 cc. of CO_2 in 24 hours (Mr. L.) was obtained in the case of a boy who for 3 weeks had lived on a strongly acid diet consisting of eggs, bread, cheese, milk, and oatmeal; the highest 362 cc. was that obtained in the case of Mr. McL. who was taking a diet composed chiefly of green vegetables, fruit, and potatoes, cream, and butter, with a little bread.

It has long been known that the reaction of the urine changes frequently during the course of the day, the "alkaline tide" which appears at varying times after eating having been a fruitful subject for student demonstrations for several decades.

In order to determine whether the variations in carbon dioxide excretion during the day bear any relation to the taking of food, and incidentally to the changes in hydrogen ion concentrations of the urine we have carried on a series of observations on the 3 hour urines of two subjects who have eaten acid-high proteins, and an alkaline vegetarian diet. In one case observations are also included when the subject was taking an ordinary mixed diet, while in the case of the other subject we have introduced in the table two experiments in which the intake of the water was greatly increased.

The experimental results obtained are incorporated in Tables II and III.

From the results presented in these tables it is apparent that the excretion of carbon dioxide, when observations are carried on during 3 hour periods, varies with the reaction of the urine in approximately the same manner as when collections are made in 24 hour amounts.

In both subjects carbon dioxide excretion appeared to have reached a maximum in the period between 7.00 and 10.00 p.m. This phenomenon is especially striking during the days in which the alkaline vegetarian diet was given, although it is still to be seen in the days during which the food was of a more acid nature.

In as far as one can judge by the rather limited number of determinations the total carbon dioxide in the 24 hour urine from a subject taking the ordinary mixed diet may vary from 20 to 211 cc.

depending on what combinations of acid and basic foods happen to be chosen by the individual.

The presence of sodium bicarbonate in considerable amounts in the strongly alkaline urine of the herbivora and of human vegetarians has long been recognized. In view of our results we wish to call attention to the probable importance of bicarbonate in determining the reactions of neutral and amphoteric human urines.

A STUDY OF THE NEPHELOMETRIC VALUES OF CHOLESTEROL AND THE HIGHER FATTY ACIDS.

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The nephelometric method of fat determinations was developed and extensively applied by Bloor.[1] His work was limited to blood fat. Recently Sharpe,[2] applying the principles laid down by Bloor, described a method for the determination of the higher fatty acids in feces. But neither of these authors investigated the intensity of the turbidity produced by each ingredient of the fatty extract made from either blood or feces. They evidently take it for granted that each ingredient produces an equal degree of turbidity. Bloor, for instance, determines the fatty acids in blood indirectly from a solution which contains the fatty acids and cholesterol. He estimates the cholesterol content independently by the colorimetric method, subtracts this amount from the sum obtained by the nephelometric method, and considers that the difference represents the amount of the fatty acid content.

Before adopting the nephelometric method for general use it was, to my mind, important to investigate certain points which are of theoretical interest in themselves, and which influence the accuracy of the results. In the present paper I wish to consider the following problems: (1) Does the concentration of the acid used to liberate the fatty acids and develop the turbidity, have any influence upon the nephelometric value of the fatty acids? (2) Is the nephelometric value the same for oleic, palmitic, and stearic acids and cholesterol? Bloor emphasizes the fact that the degree of turbidity changes rapidly, especially in the first few minutes, and arbitrarily advises that comparison be made

[1] Bloor, W. R., *J. Biol. Chem.*, 1914, xvii, 377.

[2] Sharpe, J. S., *Biochem. J.*, 1917, xi, 96.

against the standard within 5 to 10 minutes after the turbidity develops. This brings up the third question—whether this rapid change takes place to the same degree, within the same length of time, in the case of each of the substances under investigation.

Methods.

Fatty acids (Merck) in 0.1 per cent alcoholic solution were used as stock solutions. The use of an ether and alcohol mixture (Bloor) was purposely omitted. Precipitation of palmitic and stearic acids was prevented by neutralizing the solution with NaOH. Hydrochloric acid was used to develop the turbidity. The latest model Kober nephelometer with semicircular field was used in comparing the different suspensions. The total volume of each sample was approximately 50 cc., which includes 10 cc. of dilute HCl, 36 cc. of distilled water, and 4 cc. of an alcoholic solution of the fatty material. An example of the procedure by which all samples were prepared is given below. 2 cc. of the oleic acid stock solution, representing 2 mg. of oleic acid, were measured into a 150 cc. beaker. To this was added first, 2 cc. of 95 per cent alcohol, and then 36 cc. of distilled water with continual stirring. Finally, the 10 cc. of HCl were added drop by drop from a pipette, stirring the mixture constantly. Time was noted and counted from the last drop of acid added.[3]

The first problem was to determine the optimum concentration of HCl in the final 50 cc. volume, so that we should always be working under the most advantageous conditions (Table I). Since we wished in the same experiments to control the change in intensity during varying intervals of time, it became necessary to use a permanent standard. As is well known, the substances under investigation do not form permanent standards, and two of them (oleic and palmitic acids) show confusing tints of yellow or blue. A permanent standard, suitable for this particular phase of the investigation, was constructed by covering the mirror with a grayish white paper, fixing the mirror firmly so that the intensity of the field should be constant, placing a colorimetric cup with a blue filter bottom on the left (standard) side, and setting the scale at 50 mm.

DISCUSSION.

In regard to the optimum acid concentration, it is shown in Table I that a 0.2 N solution was most desirable in developing turbidity from cholesterol, stearic acid, and oleic acid. Our

[3] Before every reading it was necessary to make sure that there were no air bubbles on the under surface of the plunger. If any had collected they were eliminated by lowering the cup below the bottom of the plunger, and raising it again, very slowly.

failure always to produce with certainty an even suspension of palmitic acid made it necessary to abandon investigations on this acid except in the series 0.2 N—the optimum concentration for the other substances. A record of palmitic acid precipitated in this concentration is included in order to complete the table.

The changes of turbidity in the different acid concentrations are suggestive of a certain connection between the acid concentration and the form of suspension. In all instances the weakest turbidity was shown in the most concentrated solutions

TABLE I.

Turbidity produced by		Cholesterol.					Stearic acid.					Oleic acid.					Palmitic acid.
Amount, *mg*.....		1					1					2					1
Concentration of N HCl in final (50 cc.) volume.		1.0	0.4	0.2	0.1		1.0	0.4	0.2	0.1		1.0	0.4	0.2	0.1		0.2
Average reading of four determinations.	Time, *min.*	5 61.4	54.7	**52.5**	54.0		75.4	69.4	**67.4**	69.5		76.0	70.2	**65.4**	66.6		100.4
		10 61.4	54.5	**52.4**	53.5		77.5	71.9	**68.1**	71.2		94.3	77.1	**70.6**	71.1		100.5

after a lapse of 5 minutes. Cholesterol shows practically no change in intensity during the intervals of 5 to 10 minutes; stearic acid undergoes considerable change, while the greatest change was noted in the case of oleic acid which, in the 1.0 N solution, showed a difference of 18 mm. between the readings of 5 and 10 minutes. During the short intervals shown in the above table we find, therefore, that in the optimum concentration only the oleic acid showed a marked decrease in intensity. This rapid change between the intervals may be explained by the influence of the acid concentration upon the aggregation of the suspended particles, which decreases their power of reflecting light. If we compare readings of the series of optimum concentrations only, we find that the nephelometric value decreases in the order of cholesterol, stearic acid, palmitic acid, oleic acid, assuming that *the expression nephelometric value means the tur-*

bidity produced by a given amount of a substance compared in the nephelometer to a given standard within a given length of time.

Since different substances show differing nephelometric values and since oleic acid has been generally used as a standard in the nephelometric method of blood fat determinations (Bloor), it was important to determine the nephelometric values of the fatty substances occurring in blood against an oleic acid standard, in order to bear out the results noted in Table I. In the following experiments the optimum acid concentration was used in developing the turbidity.

The results in Table II show how fallacies may arise in comparing the turbidity of substances with different nephelometric

TABLE II

		Cholesterol.				Stearic acid.			
		Readings.*	Amount.			Readings.*	Amount.		
			Taken.	Found.	Error.		Taken.	Found.	Error.
		mm.	*mg.*	*mg.*	*per cent*	*mm.*	*mg.*	*mg.*	*per cent*
Average readings of four determinations.	Time, *min.* 5	66.8	1.0	2.09	109.0	79.7	1.0	1.75	75.0
	Time, *min.* 10	59.8	1.0	2.31	131.0	73.9	1.0	1.89	89.0

* 2 mg. of oleic acid, set at 70 mm., were used as a standard.

values. A method applied in such circumstances for the determination of quantities of different substances with varying nephelometric values is useless.

To find out whether the nephelometric value of a mixture of two substances is equivalent to the sum of the nephelometric values of the ingredients, mixtures of each substance were prepared with oleic acid. Oleic acid was a constant ingredient of these mixtures since its use gave a yellowish tint comparable to that of the standard, and its presence produced a combination of fatty material closely approximating that which is normally present in blood fat. Results of these determinations are given in Table III.

We find that the sum of the nephelometric values of the ingredients of a mixture actually is expressed in the nephelometric value of the mixture as a whole. For example, the nephelometric value of cholesterol, previously determined against oleic acid as a standard (Table II) is 2.09. Turning to the nephelometric value of the cholesterol mixture (2.93) and subtracting from this sum the nephelometric value of the oleic acid content (1.0) we find the nephelometric value of cholesterol to be 1.93—a very close agreement. Similarly, in the case of stearic acid, the nephelometric value 1.75 becomes 1.74 when determined from the mixture. We may apply these principles to the determination of the nephelo-

TABLE III.

Time.	Cholesterol mixture.				Stearic acid mixture.				Palmitic acid mixture.				Remarks.
	Readings.*	Amount.		Error.	Readings.*	Amount.		Error.	Readings.*	Amount.		Error.	
		Taken.	Found.			Taken.	Found.			Taken.	Found.		
min.	*mm.*	*mg.*	*mg.*	*per cent*	*mm.*	*mg.*	*mg.*	*per cent*	*mm.*	*mg.*	*mg.*	*per cent*	Each mixture consists of 1 mg. of the named substance added to 1 mg. of oleic acid.
5	47.7	2.0	2.93	46.5	51.0	2.0	2.74	37.0	51.6	2.0	2.71	35.0	
10	36.4	2.0	3.01	50.5	49.2	2.0	2.85	42.5	50.1	2.0	2.79	39.5	

* Average taken from four determinations. 2 mg. of oleic acid, set at 70 mm., were used as a standard.

metric value of a substance which presents difficulties when used alone but whose nephelometric value may be determined from a mixture. This was true, in our case, of palmitic acid. The nephelometric value of 1 mg. of a substance compared to oleic acid as a standard and determined by the methods of procedure outlined above is as follows: cholesterol 1.93, stearic acid 1.74, palmitic acid 1.71, oleic acid 1.0. In other words, the turbidity of 1 mg. of cholesterol, compared in the nephelometer, is equal to the turbidity of 1.93 mg. oleic acid.

I wish to emphasize the phrase "compared in the nephelometer" because if we compare suspensions, one containing 1 mg. of oleic acid and the second containing 1 mg. of cholesterol, stearic acid, or palmitic acid, and simply glance down upon them, we receive a misleading impression; *i.e.*, the oleic

acid will seem to show a stronger turbidity. Now, if we make a mixture of 1 mg. of oleic acid and 1 mg. of stearic acid and compare this with a suspension of 2 mg. of oleic acid, our casual judgment of the degree of turbidity will be borne out by readings made in the nephelometer. In the first instance our judgment was contrary to the nephelometric findings; in the second it was in accordance with them, showing the unreliability of readings made outside the instrument.

The importance of the results discussed in the present paper on nephelometric fat determinations in general will be reserved for a later presentation.

SUMMARY.

The expression "nephelometric value" of a substance is defined.

Investigations were conducted with cholesterol, stearic acid, palmitic acid, and oleic acid and the following points noted:

The nephelometric value of these substances is influenced by the acid concentration of the suspension.

The optimum acid concentration is that which produces the highest nephelometric value with the least change within specified intervals of time. This change does not take place with the same rapidity and to the same degree in each case.

The nephelometric values of cholesterol, stearic acid, palmitic acid, and oleic acid are different.

THE METABOLISM OF THE MUSTARD OILS.*

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Plant materials containing the mustard oils have been used by man as a food in practically all countries for ages. The peoples of the Mediterranean countries in particular are fond of onions, garlic, leeks, etc., as constituents of their food. These plants together with the turnip, cabbage, radish, and mustard are used generally throughout the world either as staple articles of food, or for their condimental value. In the feeding of domestic animals, rape, turnips, cabbage, rape seed cake, and Indian mustard cake form a very important part of the ration. The last two feeding materials have been confined almost entirely to Europe and India. In this country there is a considerable quantity of wild mustard separated from wheat that is used for stock food. Winton (1) estimates that from the Minneapolis mills alone more than 100 carloads per year are obtained. The use of these mustard cakes has been accompanied by considerable doubt as to their suitability for animal consumption. There is a widespread feeling that they are more or less toxic, and numerous cases of diarrhea, abortion, fever, and even death have been attributed to their use.

With a view to throwing some light on the question of the toxicity of rape and mustard Ulbricht (2) carried out several feeding experiments on sheep, a calf, and milch cows. In most of the experiments these animals showed no ill effects from the consumption of large amounts of mustard and rape meal, but nevertheless the conclusion was drawn that these materials must be fed with caution.

Numerous cases of sheep and cattle poisoning due to the eating of rape cake appeared in two provinces of Holland in the years 1897 and 1898. Sjollema (3) investigating this cake found an extremely high percentage (0.8 per cent) of mustard oil present. An emulsion of starch, gum arabic, and 0.200 gm. of mustard oil, obtained from black mustard, when introduced into the stomach of rabbits invariably caused their death. Cramps, exudation of blood, and necrosis of the intestinal wall, together with marked edema of the abdominal organs, were the symptoms accompanying the death of the animals.

Moussu (4) found that cattle could take by tube single doses of from 1.2 to 3.0 gm. of allyl isothiocyanate without permanent injury, but that,

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when the dose was increased to 5.0 gm., death ensued within 12 hours with all the symptoms of acute enteritis. The animals seemed to be able to eat large quantities of rape cake without injury, a goat consuming per 100 kilos of body weight enough rape cake to yield 14.2 gm. of crotonyl isothiocyanate.

The tolerance of guinea pigs toward allyl isothiocyanate seems to be very considerable. For 82 days Meyer (5) daily injected subcutaneously a mixture of olive oil and allyl isothiocyanate containing 0.002 gm. of the mustard oil; then, after an interval of 23 days 0.002 gm. daily for 12 days longer. At the end of this time the animal was killed and examined. The liver and kidneys were slightly swollen and discolored, but showed no necrosis of the cells.

A more extensive investigation on the physiological effects of allyl isothiocyanate and allyl sulfide has been carried out by Carlier (6). He injected varying doses of the synthetic mustard oils dissolved in olive oil into the jugular vein of rabbits and noted the effect on the blood pressure, heart beat, and respiration. Both drugs produced a paralysis of the respiratory and vasomotor centers, muscular spasms, and a lowering of the body temperature. The effect began within a few seconds and continued for many minutes. Both drugs are alike in their general behavior, but the allyl isothiocyanate is more powerful than the allyl sulfide. The lethal dose of the former was found to be 0.012 cc. per kilo of body weight and 0.03 cc. for the latter.

A case of onion poisoning in cattle was reported by Goldsmith (7) in which two out of nine animals affected died. A postmortem examination showed inflammation of the rumen and intestines, an enlarged liver, badly discolored kidneys, and a strong odor of onions about the whole carcass.

In speaking of the toxicity of the mustard oils,[1] it is necessary to specify the particular oil used, as the generic term mustard oils includes a large number of compounds, differing widely in their chemical composition and physiological behavior. Stein (8) found that crotonyl isothiocyanate (C_4H_7CNS) was only one-fifth as toxic to rabbits as its homologue allyl isothiocyanate (C_3H_5CNS), and Carlier showed the latter oil to be two and one-half times as toxic as allyl sulfide $(C_3H_5)_2S$, the chief mustard oil of the onion. It is likewise undoubtedly true that the oils are much more injurious when introduced into the intestinal tract in the free state than when taken in the combined or glucoside form, in which they occur in the seed of the plant.

The glucosides (9) of the mustard oils are compounds of the general formula

[1] Mustard oil is used in this paper as a general term to signify both allyl sulfide and allyl isothiocyanate.

$$\begin{array}{l} O-SO_2-O-R \quad (K, C_{16}H_{24}O_5) \\ \quad\quad\quad\

without serious injury. On the other hand the presence of a large amount of glucoside with a particularly active enzyme may be responsible for the cases of poisoning reported. The age, treatment of the cake, and conditions obtaining in the digestive tract all influence the activity of the enzyme.

There is very little evidence in the literature as to what chemical changes, if any, the mustard oils undergo in the course of metabolism. That the body handles these compounds with great difficulty is evident from the experiments cited. Some of the investigators mentioned report that the organs, skin, urine, and feces of animals poisoned by mustard oils reek with their odor. An odor in the breath and a taste in the milk after the ingestion of onions indicate that the animal does not destroy them to any extent.

EXPERIMENTAL.

To throw some light on what chemical changes the mustard oils undergo in the body, the experiments that follow were designed. Since all the mustard oils contain sulfur, it was hoped that a study of the sulfur excretion would give some indication of the metabolic changes involved. If the mustard oils are excreted by way of the kidneys, an increase in the total sulfur and a change in its partition should be observed.

Sulfur in the Urine.

The total sulfur and its distribution as sulfates, ethereal sulfates, and unoxidized sulfur were determined in a preliminary period, during the ingestion of the mustard oil, and in a period immediately following the addition of the mustard oil to the ration. The well known methods of Benedict (11) and Folin (11) were used in these analyses. In two of the experiments, the quantity of volatile sulfur in the urine was determined. For this purpose 400 cc. of urine, to which 10 cc. of phosphoric acid had been added, were distilled with steam, and the distillate (400 cc.) was collected in alkaline permanganate in much the same way as mustard oils have been determined (12). A trial with allyl isothiocyanate added to urine gave a quantitative recovery.

Sulfur in the Feces.

In two of the experiments, the total sulfur excreted in the fore period, mustard oil period, and after period was determined on the dried feces by the sodium peroxide fusion method.

Volatile sulfur was determined in two of the experiments for nearly the entire duration of the experiments, and on single days in a third experiment. To the wet feces (25 to 50 gm.) 10 cc. of phosphoric acid and 200 cc. of water were added and the mixture was distilled with steam, as in the determination of volatile sulfur in the urine.

Ration and Animals.

In order to eliminate as far as possible the influence of sulfur from other sources than the mustard oil the following basal ration was fed daily: starch, 60 calories per kilo of weight; salt mixture, 10 gm.; filter paper, 10 gm.; distilled water, 2,000 cc. The mustard oil was added to this basal ration in the amounts indicated in the tables. The oils used were all synthetic compounds.

As the starch was found to contain some sulfates, in two of the experiments it was extracted from 24 to 48 hours with 2 per cent HCl and then washed with distilled water until practically free from chlorides. Not all of the sulfur in the starch could be completely removed even with this careful washing.

The composition of the salt mixture was as follows:

	gm.
NaCl	50
K_2HPO_4	120
$Ca_3H_2(PO_4)_2$	30
$MgCl_2.6H_2O$	40
Ferric citrate	10

The filter paper was extracted with 2 per cent HCl for 5 or 6 days and then washed free from chlorides as in the case of starch.

Previous experiments in this laboratory (13) have shown that a pig will eat the above ration readily for 60 days or longer, without any particular loss of weight. A preliminary attempt to use a goat on a more varied ration than the above failed completely because of the fastidious appetite of this animal. The pigs used in these experiments were all vigorous animals and retained their appetites throughout the experiment.

A preliminary feeding of from 10 to 15 days on the basal ration was carried out before beginning the collection of urine and feces for analysis.

Feeding of Allyl Isothiocyanate.

Experiment I.—A pig weighing 76 pounds at the beginning of the experiment and 77 pounds at its close was used. The starch, salts, and filter paper were scalded with about 300 cc. of water and after cooling, the remainder of the 1,000 cc. of water was added. When cold a solution of mustard oil (2 gm.) in olive oil (18 gm.) was added to the starch until this contained the quantity of pure mustard oil indicated in the table. This starch-mustard oil mixture was about the consistency of a thick soup and was eaten by the animal usually in 5 minutes.

During the latter half of the experiment, butter fat was added to the ration, as it was thought that this might aid in the absorption of the mustard oil. There is no evidence in the data, however, that this is the case.

A survey of Table I shows: (1) A gradual increase in the total sulfur excreted and that this higher excretion continues for 12 days after the feeding of the mustard oil has ceased. This indicates the difficulty with which the body disposes of the mustard oil. (2) This increase is chiefly in the unoxidized sulfur of the urine. Here is evidence that the mustard oils are not oxidized to simple compounds in their passage through the body. They are not excreted unchanged, however, as will be shown later. (3) That the increase in total sulfur could not have arisen from the catabolism of body protein is indicated by the constancy of the figures for nitrogen excretion.

Experiment II.—A larger animal weighing 95 pounds at the beginning and 98 pounds at the close of the experiment was used.

The mustard oil (10 gm.) was dissolved in alcohol (100 cc.). On the 1st day of the mustard oil period, a suspension of the oil in 1 per cent starch solution was given to the animal by tube after it had eaten about half of the morning feeding; but the pig immediately regurgitated most of it. A second attempt in the afternoon, using a smaller quantity of oil, was no more successful. The oil was then added to each feeding as in Experiment I. This animal showed less liking for the starch-mustard oil mixture and after 12 days ate it only with great reluctance.

TABLE 1.—*(Results of Experiment 1.)*

Day.	Ration.	Volume of urine.	Sulfur distribution as $BaSO_4$.				Nitrogen
			Total.	Sulfates.	Ethereal.	Unoxidized.	
Period 1. Fore period.							
	gm.	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1-3 average per day	Starch 500	1,603	1.023	0.719	0.121	0.183	—
4	" 500	1,655	1.028	0.743	0.109	0.180	—
Average per day		1,616	1.024	0.724	0.118	0.182	
Period 2. Allyl isothiocyanate.							
5	Starch 500						
6-7 average per day	Allyl isothiocyanate 0.240 Starch 500	1,395	1.024	0.600	0.138	0.286	1.77
8-11 " " "	Allyl isothiocyanate 0.223 Starch 500	1,432	1.100	0.672	0.127	0.301	1.76
12-15 " " "	Allyl isothiocyanate 0.241 Starch 400 Butter fat 60	1,564	1.382	0.830	0.157	0.395	2.04
16	Allyl isothiocyanate 0.365	1,149	1.236	0.708	0.071	0.457	1.83
	Same as 12th-15th days	1,300	1.560	0.889	0.069	0.602	1.91
Average per day		1,368	1.271	0.749	0.114	0.408	1.89
Period 3. No allyl isothiocyanate.							
17	Starch 400 Butter fat 60	1,460	1.492	0.919	0.075	0.498	2.13
18-20 average per day	Same as 17th day	1,522	1.241	0.742	0.094	0.405	1.68
21-24 " " "	Starch 400 Butter fat 120	1,224	1.290	0.738	0.121	0.431	2.02
25-28 " " "	Starch 400	1,666	1.144	0.642	0.090	0.412	2.12

As in Experiment I, the data show an increase in the total sulfur excretion and the continuance of this increase for about 10 days after discontinuing the mustard oil feeding. It is evident that the fore period was too short, as the animal had not reached a constant level of sulfur excretion. Had this been longer, it is probable that the increase in Period 2 would have been more pronounced. In this experiment the increase is not confined as markedly to the unoxidized sulfur, as in Experiment I. The high figures obtained for the 7th day are probably due to the disturbance of the 6th.

The amount of volatile sulfur was determined in this case and shows a slight increase during the mustard oil feeding, due possibly to some of the mustard oil being excreted in a volatile form in the urine. There was no odor of mustard oil about the urine, however, and the quantity of volatile sulfur obtained is insignificant as compared to the quantity ingested. It is therefore not excreted in an unchanged form. It is possible that some such reaction has taken place as occurs between allyl isothiocyanate and ammonia in the formation of thiosinamine (C_3H_5–$NH(NH_2).CS$), a non-volatile substance, the toxicity of which is very much less than the mustard oil itself.

Feeding of Allyl Sulfide.

Experiment III.—Weight of animal 58 pounds.

Allyl sulfide has been found (6) to be much less toxic than allyl isothiocyanate. This is very evident from the much larger quantities of mustard oil that can be ingested without injury. The taste and odor of the sulfide is very much less intense than that of the cyanate.

An increase of about 20 per cent in the total sulfur is observed. The increase continued to the end of the experiment, which was discontinued 3 days after the end of the mustard oil period because of long confinement and a tendency toward diarrhea induced probably by the butter fat. The unoxidized sulfur shows a greater increase than the total, while the sulfates show an actual decrease. This addition of butter fat may have decreased the oxidation processes in the body and thus reduced the sulfates

TABLE II.—(*Results of Experiment II.*)

Day.	Ration.		Volume of urine.	Sulfur distribution as $BaSO_4$.					Nitrogen.
				Total.	Sulfates.	Ethereal.	Unoxidized.	Volatile sulfur.	
		gm.	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Period 1. Fore period.									
1–2 average per day	Starch	650	1,800	1.328	0.536	0.232	0.559		2.04
3–4 " " "	"		1,795	1.220	0.447	0.165	0.608		1.85
5 " " "	"		1,760	1.091	0.399	0.103	0.589	0.008	1.38
Average per day of 3–5			1,791	1.177	0.431	0.141	0.605		1.83
Period 2. Allyl isothiocyanate.									
6	Starch	650							
	" allyl isothiocyanate	0.342	*						
7	" " "	0.195	1,680	1.782	0.703	0.359	0.720	0.019	2.41
8–9 average per day	" " "	0.195	1,570	1.200	0.476	0.179	0.545	0.035	1.73
10–12 " " "	" " "	0.358	1,775	1.447	0.533	0.159	0.756	0.032	1.81
13–14 " " "	" " "	0.390	1,800	1.577	0.570	0.192	0.815	0.048	1.97
15–17 " " "	" " "	0.293	1,620	1.409	0.599	0.129	0.681	0.030	2.12
Average per day for period			1,690	1.446	0.563	0.178	0.705		1.96
Period 3. No allyl isothiocyanate.									
18–20 average per day	Starch	650	1,870	1.280	0.610	0.089	0.581	0.012	1.91
21–23 " " "	"		1,695	1.227	0.547	0.171	0.509	0.011	2.02
24–26 " " "	"		1,975	1.033	0.413	0.125	0.495	0.008	1.71
27–30 " " "	"		1,755	0.840	0.258	0.099	0.483	0.011	1.53

* Vomited; urine discarded.

TABLE III.—(*Results of Experiment III.*)

Day.	Ration.	Volume of urine.	Sulfur distribution as $BaSO_4$				Nitrogen
			Total.	Sulfates.	Ethereal.	Unoxidized.	
Period 1. Fore period.							
		gm.	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1	Starch	400	1,535	0.972	0.622	0.126	0.224	1.69
2–4 average per day	"	400	1,530	1.024	0.728	0.080	0.212	1.69
5	"	400	1,480	0.952	0.666	0.058	0.228	1.52
Average per day for period			1,515	0.981	0.672	0.088	0.221	1.60
Period 2. Allyl sulfide.							
6	Starch	400	1,300	0.820	0.340	0.194	0.286	1.30
	Allyl sulfide	0.480						
7–9 average per day	Starch	400	1,480	1.076	0.572	0.056	0.448	1.50
	Allyl sulfide	0.760						
10	Starch	400	1,515	1.188	0.446	0.132	0.610	1.50
	Allyl sulfide	0.804						
11–13 average per day	Starch	400	1,230	1.296	0.268	0.138	0.890	1.87
	Butter fat	100						
	Allyl sulfide	1.005						
14	Same as 11th–13th days		1,600	1.432	0.464	0.084	0.884	1.98
Average per day for period			1,394	1.173	0.419	0.110	0.644	1.65
Period 3. No allyl sulfide.							
15	Starch	400	1,350	1.488	0.420	0.200	0.868	—
	Butter fat	100						
16	Same as 15th day		1,320*					
17	" " 15th "		1,400	1.496	0.678	0.108	0.710	—

*Urine lost after collection.

and increased the unoxidized sulfur arising from the metabolism of body protein.

Experiment IV.—Weight of animal 54 pounds.

A long preliminary period on starch and salts before the collection of urine and feces had brought this animal to a low level of sulfur excretion. The mustard oil was given to the animal by tube after about half of the food had been consumed. The animal did not seem to be at all disturbed by this procedure, as it consumed the remainder of the feeding immediately and did not regurgitate any of it.

The increase in the total sulfur is the greatest for any of the experiments, rising from an average of 0.451 gm. to 1.011 gm., an increase of about 125 per cent. The increase is entirely in the unoxidized sulfur and indicates that the mustard oil remains unoxidized in its passage through the animal. There was a distinct odor of mustard oil about the urine, especially toward the end of the mustard oil period. The volatile sulfur determination, however, shows clearly that only a small quantity of the mustard oil ingested was excreted unchanged. It corresponds to but a small part of the increase in the unoxidized sulfur. From this data it is logical to conclude that while the oil is not oxidized to sulfates it is changed to a non-volatile compound in its passage through the organism. Just what the nature of this change might be it is difficult to suggest. A partial oxidation to a non-volatile organic compound, such as other organic sulfides may undergo, is a possibility.

As in the case of all the other experiments, the data show a high excretion of total and unoxidized sulfur for many days after the ingestion of mustard oil has ceased. There is also a lag between ingestion and excretion. The excretion does not begin promptly, nor does it stop immediately.

The excretion of total sulfur and of unoxidized sulfur, and their relation to the ingestion of mustard oil, are shown graphically by Figs. 1 to 4 numbered to correspond with each experiment. Since there is considerable variation from day to day, the curves have been drawn to show the general trend rather than to include every point. They clearly show a slow upward trend which reaches its maximum very nearly at the end of the mustard oil feeding, and a corresponding slow fall after the in-

TABLE IV.—(*Results of Experiment IV.*)

Day.	Ration.	Volume of urine.	Sulfur distribution as $BaSO_4$.					Nitrogen.
			Total.	Sulfates.	Ethereal.	Unoxidized.	Volatile sulfur.	
			Period 1. Fore period.					
		gm.	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1–3 average per day	Starch	400	1,145	0.482	0.191	0.097	0.194		1.24
4	"		1,335	0.358	0.066	0.128	0.165	0.016	1.01
Average per day for period			1,193	0.451	0.159	0.105	0.187		1.08
			Period 2. Allyl sulfide.						
5	Starch	400							
	" allyl sulfide	0.7	1,075	0.425	0.073	0.071	0.281	0.013	0.96
6–8 average per day	" " "	1.0	1,255	0.649	0.087	0.071	0.491	0.070	1.21
9–10 " " "	" " "	1.2	1,550	1.244	0.278	0.080	0.886	0.173	1.25
11–12 " " "	" " "	1.4	1,140	1.376	0.204	0.096	1.076	0.127	1.16
13	" " "	1.1	1,105	1.485	0.160	0.074	1.251	0.272	1.15
Average per day for period			1,258	1.011	0.162	0.079	0.770		1.17
			Period 3. No allyl sulfide.						
14	Starch	400	935	1.255	0.270	0.071	0.914	0.247	0.98
15–16 average per day	"		1,105	0.998	0.217	0.063	0.717	0.157	1.01
17–18 " " "	"		1,245	0.858	0.057	0.128	0.673	0.101	0.87
19–20 " " "	"		1,120	0.705	0.011	0.109	0.585	0.066	0.82
21–22 " " "	"		1,095	0.537	0.034	0.098	0.405	0.037	0.82

gestion of mustard oil has ceased. The close parallelism existing between the curves for total sulfur and unoxidized sulfur, is quite evident and points to the excretion of the mustard chiefly as unoxidized compounds.

Sulfur in the Feces.

It has been said (14) that the odor of mustard oils can be recognized "in the excreta of persons who have consumed the

TABLE V.

Volatile Sulfur in Wet Feces.

Experiment No.	Ration.	Time.	Weight of dry feces.	Volatile sulfur as $BaSO_4$.	Sulfur in mustard oil fed as $BaSO_4$.
		days	*gm.*	*gm.*	*gm.*
I	Starch and allyl isothiocyanate.....	7	102	0.167	3.823
	" butter, and allyl isothiocyanate.....	5	63	0.256	4.305
	" butter.....	8	150	0.197	0.000
	"	4	54	0.064	0.000
II	" and allyl isothiocyanate.....	12	171	0.174	7.830
	"	7	93	0.093	0.000
IV	"	1	54	0.049	0.000
	" and allyl sulfide.....	2	46	0.076	4.10

oil in the form of a sauce into which a chive or onion has been dipped." In the feces of these four animals there was at no time an odor that could be clearly recognized as that of the mustard oil. The chemical analysis shows no evidence of any appreciable amount of unabsorbed mustard oil. The volatile sulfur determined would include unchanged mustard oils, hydrogen sulfide, and any other sulfur body volatile with steam. This total volatile sulfur is so small in comparison to the amounts of mustard oil ingested as to indicate clearly the absorption from the intestinal tract of the major part of the oil. In Experiment I there is only 0.42 gm. of volatile sulfur excreted during the mustard oil period of 12 days and 0.26 gm. in the 12 days of no mustard oil that follow. The difference, 0.16 gm., is insignificant as compared to the quantity (8.12 gm.) of mustard oil in the ration.

In Experiment IV, Table V, where the largest amount of mustard oil was fed, the 2 days of the mustard oil period average 0.038 gm. per day, while the last day of the fore period shows a larger figure, 0.049 gm. During the 2 days the animal received enough mustard oil to equal 4.10 gm., calculated as barium sulfate, showing very conclusively that the mustard oil was not excreted in the feces.

TABLE VI.

Total Sulfur in Feces.

Experiment No.	Ration.	Time.	Weight of dry feces.	Total sulfur as $BaSO_4$.	Sulfur per gm. of feces as $BaSO_4$.
		days	*gm.*	*gm.*	*gm.*
II	Starch	8	109	2.33	0.0214
	" and allyl isothiocyanate	12	271	5.718	0.0211
	"	9	167	2.937	0.0176
IV	"	4	104	2.328	0.0223
	" and allyl sulfide	9	252	5.292	0.0210
	"	10	316	6.541	0.0207

The determination of the volatile sulfur in the wet feces does not preclude the possibility of the destruction of the mustard oil by bacteria and other organisms and the subsequent excretion in the feces of the products formed. Table VI gives the total sulfur in the dried feces for Experiments II and IV. From Experiment II it might appear that a large increase of sulfur

TABLE VII.

Summary of Mustard Oils Fed and Increase in Excretion of Sulfur Obtained in the Urine.

	Allyl isothiocyanate as $BaSO_4$.		Allyl sulfide as $BaSO_4$.	
Experiment No.	I	II	III	IV
No. of days in Periods 2 and 3	24	21	12	18
Total sulfur excreted in Periods 2 and 3, *gm.*	30.204	27.972	15.032	16.547
Calculated excretion from average per day of Period 1, *gm.*	24.576	24.717	11.772	8.118
Increase due to mustard oils, *gm.*	5.728	3.255	3.260	8.429
Quantity of mustard oil fed, *gm.*	3.444	3.318	3.577	10.0
Sulfur content of mustard oil, *gm.*	8.128	7.830	7.512	20.480
Mustard oil sulfur accounted for, *per cent.*	70	41	43	41

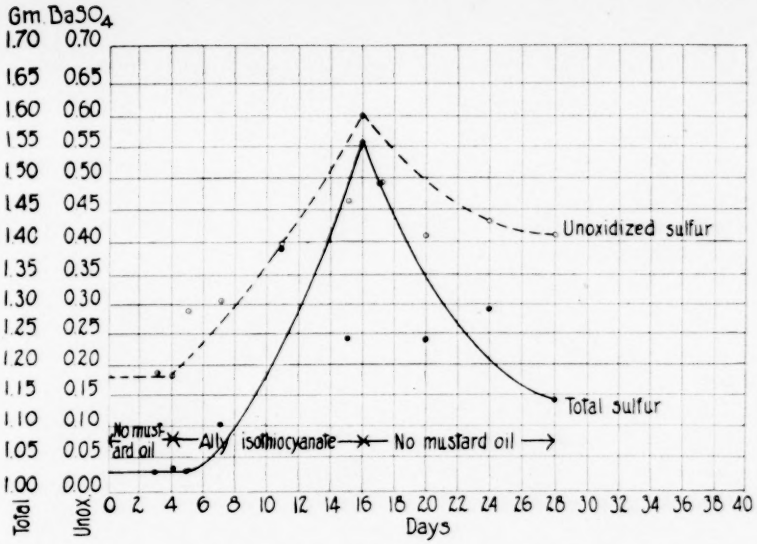

FIG. 1. Experiment I. Sulfur excretion as related to the ingestion of allyl isothiocyanate. A close parallelism exists between the two curves in all the figures, indicating little or no oxidation of the mustard oils.

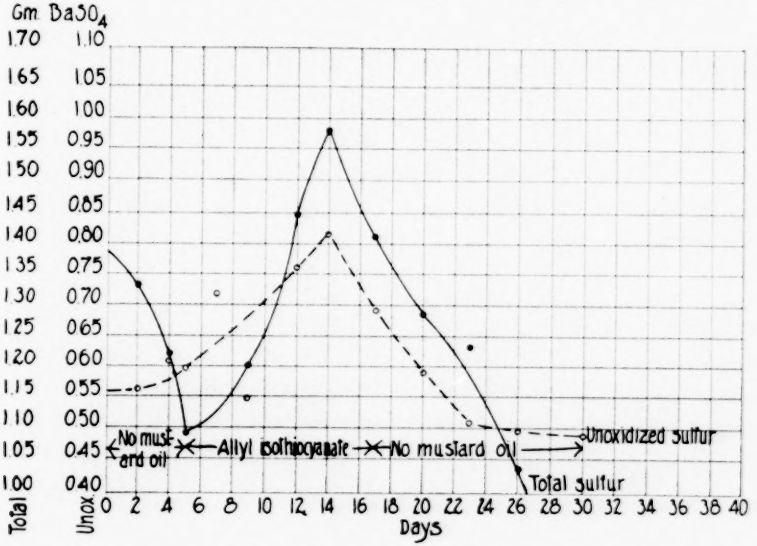

FIG. 2. Experiment II. The same relations are shown as in Fig. 1 but with another animal.

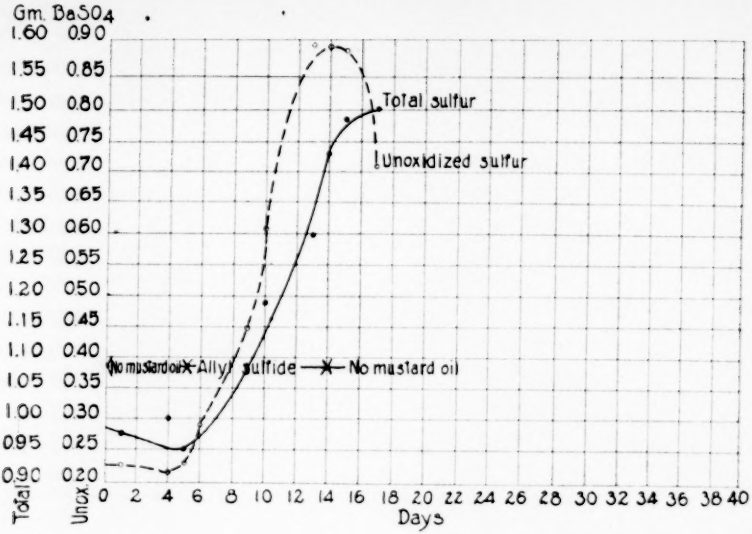

FIG. 3. Experiment III. The effect of feeding allyl sulfide on the sulfur excretion.

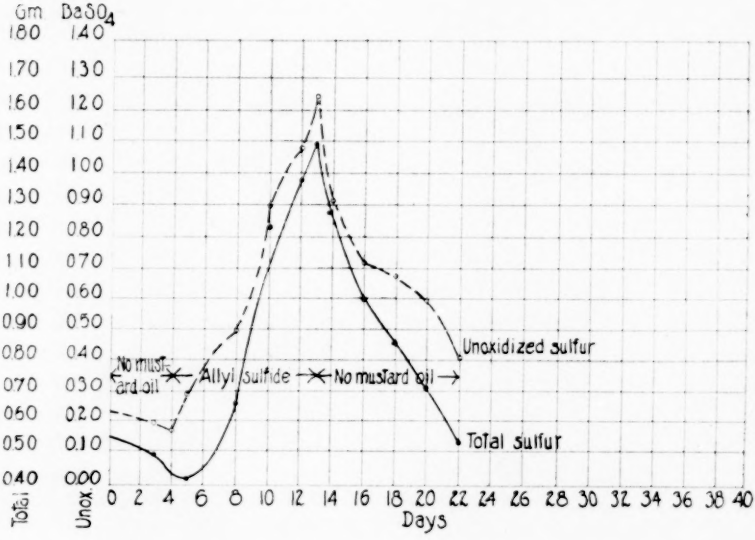

FIG. 4. Experiment IV. A longer and more complete experiment than is shown in Fig. 3. Note the *slow rise* of the curves with the ingestion of mustard oil and the *gradual decline* after ingestion has ceased.

occurs in the feces of the mustard oil period; but in Experiment IV the output is even greater in the after period than in the mustard oil period. A closer examination discloses a coincident increase in the weight of feces excreted, with a very constant amount of sulfur per gm. of feces. The quantity of sulfur excreted is therefore not related to the ingestion of mustard oil, but only to the weight of feces excreted. This supports the conclusion, deduced from the data for volatile sulfur, that the mustard oils are not excreted in the feces.

In Table VII are summarized the results obtained in the experiments. The increased sulfur excreted, due to the feeding of mustard oils, is shown in comparison with the quantity of sulfur contained in the oils. In Experiment I the increased excretion amounts to 5.728 gm. as compared to 8.128 gm. ingested, equivalent to about 70 per cent recovery of the sulfur content of the mustard oils. For the other three the percentage is in the neighborhood of 42 per cent. These figures represent a minimum recovery, as a much higher percentage could be obtained by taking the last days of the fore period and a longer after period as a basis for calculation.

The mustard oil unaccounted for is either still retained in the body or else has been excreted through the lungs and the skin.

SUMMARY.

1. The addition of allyl isothiocyanate, 0.2 to 0.4 gm. per day and allyl sulfide, 0.5 to 1.0 gm. per day, to a starch ration produces a gradual increase in the total sulfur excreted in the urine, and a continuance of this high increase for 10 to 12 days after the ingestion of the mustard oils has ceased. This points to a very slow metabolism of the oils and the inability of the body to dispose of these oils readily.

2. The increased sulfur excretion is confined mainly to the unoxidized part of the sulfur excreted. The curves for total sulfur and unoxidized sulfur rise and fall together.

3. Although the mustard oils are not oxidized in their passage through the animal, they are not eliminated unchanged as there is little or no mustard oil in the urine. It is possible that they are converted into some non-volatile less toxic substance. When

large quantities of allyl sulfide are ingested, an odor of mustard oil may be detected from the urine, but the amount thus excreted is small.

4. That the mustard oils are absorbed from the digestive tract is shown by the fact that there is no particular increase in the volatile sulfur or total sulfur in the feces.

5. The data here presented show that, calculated on a minimum basis, from 40 to 70 per cent of the above quantities of mustard oils are excreted in the urine. The remainder may be excreted through the lungs and the skin.

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14. Johnson and Church, quoted by Carlier and Evans (6), 329.

EXPERIMENTAL STUDIES ON CREATINE AND CREATININE.

VIII. THE ALLEGED EXOGENOUS ORIGIN OF URINARY CREATINE IN THE PROTEIN OF THE DIET.*

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The study of metabolism in recent years has led to the formulation of three chief theories in explanation of the presence of creatine in the urine. The first of these to receive serious consideration was the carbohydrate deficiency theory. Catheart[1,2] early observed the potency of carbohydrates in causing the disappearance of creatine from the urine of starving man. This fact was further investigated by Mendel and Rose[3] who advanced the hypothesis that the *conversion* of creatine into creatinine was intimately associated with the oxidation of carbohydrates. Numerous investigators[4] have subsequently adduced data tending to corroborate this theory. There seems, therefore, little reason for doubting that in a great many conditions at least, the presence of creatine in the urine of adults is dependent upon a deficient supply of carbohydrate, or to a defect in the carbohydrate metabolism of the individual.

* The earlier papers of this series were published as follows: Mendel, L. B., and Rose, W. C., *J. Biol. Chem.*, 1911–12, x, 213, 255. Rose, W. C., *ibid.*, 1911–12, x, 265; 1912, xii, 73; 1916, xxvi, 331. Rose, W. C., Dimmitt, F. W., and Cheatham, P. N., *ibid.*, 1916, xxvi, 339; Rose, W. C., and Dimmitt, F. W., *ibid.*, 1916, xxvi, 345.

[1] Catheart, E. P., *Biochem. Z.*, 1907 vi, 109.

[2] Catheart, *J. Physiol.*, 1909–10, xxxix, 311.

[3] Mendel and Rose, *J. Biol. Chem.*, 1911–12, x, 213.

[4] Mellanby, E., *Lancet*, 1911, ii, 8. Benedict, S. R., and Osterberg, E., *J. Biol. Chem.*, 1914, xviii, 195. Rose, *ibid.*, 1916, xxvi, 331. Janney, N. W., Goodhart, S. P., and Isaacson, V. I., *Arch. Int. Med.*, 1918, xxi, 188.

On the other hand some investigators are of the opinion that the presence of creatine in the urine is usually or always due to an excess ingestion of protein rather than to an inadequate supply of available carbohydrate. Folin and Denis[5] offer this as the explanation of the excretion of creatine in normal children. McCollum and Steenbock[6] maintain that the feeding of certain corn products to pigs is sufficient to induce the excretion of creatine. They suggest that the arginine of the diet may be responsible, and that therefore it is perhaps not the high protein intake *per se*, but rather the high content of proteins in certain creatine precursors which is responsible for creatinuria. Denis and Kramer report that in adults with Graves' disease,[7] and in normal children[8] the creatine output can be greatly augmented by increasing the protein of the diet, while it is promptly diminished in amount, or made to disappear entirely by lowering the intake of protein. More recently Denis and Minot[9] report that diets rich in protein lead to the excretion of creatine in normal women but not in normal men. The protein of these diets was obtained largely from eggs, cheese, milk, and gelatin. If these investigators regard creatine production a property of certain proteins and not of all as indicated by McCollum and Steenbock, they make no mention of this fact. A perusal of Denis' papers would lead one to believe that any of the ordinary proteins would manifest this behavior.

Underhill[10] has suggested that creatine elimination may be intimately associated with acidosis. In an interesting series of papers he and his coworkers have adduced a great deal of evidence in support of this theory. Nevertheless they specifically state that acidosis will not account for the presence of creatine in all conditions. Underhill and Baumann[11] believe that either acidosis with no carbohydrate deficiency, or carbohydrate deficiency in the absence of acidosis may cause the elimination of creatine.

[5] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 253.
[6] McCollum, E. V., and Steenbock, H., *J. Biol. Chem.*, 1912–13, xiii, 209.
[7] Denis, W., *J. Biol. Chem.*, 1917, xxx, 47.
[8] Denis, W., and Kramer, J. G., *J. Biol. Chem.*, 1917, xxx, 189.
[9] Denis, W., and Minot, A. S., *J. Biol. Chem.*, 1917, xxxi, 561.
[10] Underhill, F. P., *J. Biol. Chem.*, 1916, xxvii, 127.
[11] Underhill, F. P., and Baumann, E. J., *J. Biol. Chem.*, 1916, xxvii, 151.

A critical examination of the three theories briefly outlined above reveals one very fundamental difference in them. The acidosis theory of Underhill[10] and the carbohydrate deficiency theory as developed by Mendel and Rose[3] agree in attributing urinary creatine in individuals on a creatine-free diet to an entirely *endogenous* source, namely, the creatine of the tissues. The theory of McCollum and Steenbock[6] and of Denis[7, 8, 9] suggests an *exogenous* origin of urinary creatine in certain amino-acids of the diet. It is universally recognized that in ultimate analysis tissue creatine must be synthesized from amino-acids, but the question at issue is: Does creatine formation proceed only so far as this substance is needed by the cells, or may the reaction be induced indefinitely by the introduction of creatine precursors? In other words, Is creatine an *anabolic* product formed for a specific purpose or function, or is it a normal product of the *catabolism* of protein, whether this protein be of tissue origin (starvation) or of food origin (high protein diet)? The latter would seem to be the conception of McCollum and Steenbock, and of Denis. Indeed, as a working hypothesis Denis states,[7] "that on the ingestion of protein some fraction of this is transformed into creatine, transported to the muscles, and there absorbed. If so much creatine is manufactured that the muscles become supersaturated, creatine is excreted by way of the kidney." Denis adds that, "It must also be borne in mind that the muscles of different individuals on the same diet may and probably do have different saturation points." Accordingly, the creatine excretion in children, and in adults with exophthalmic goiter may be due not only to the high protein of the diet, but perhaps also to a low saturation point of the muscles for creatine.

The protein theory would be the most attractive, and in many ways the simplest hypothesis offered in explanation of creatine elimination provided the experimental results upon which it is based could be substantiated beyond all possibility of doubt. We believe, however, that the proof for it is far from adequate. The results obtained by Rose, Dimmitt, and Cheatham[12] in this laboratory, in which it was clearly shown that contrary to the generally accepted idea, protein feeding in fasting man promptly

[12] Rose, Dimmitt, and Cheatham, *J. Biol. Chem.*, 1916, xxvi, 339.

causes the reduction or disappearance of urinary creatine, at once present difficulties in the acceptance of the Denis hypothesis. It is difficult to understand how the feeding of such enormous quantities of protein (thirty to forty eggs) to fasting men can result in the entire disappearance of creatine, if this substance is being continually formed from the amino-acids of the diet. One must assume that either the tissues have become remarkably deficient in creatine during the few days of abstinence from food, or else that the ability of the muscles to absorb creatine has been marvelously increased. For the latter assumption there certainly is no evidence, while the investigations of Mendel and Rose,[13] Myers and Fine,[14] and others indicate that far from there being a deficient amount of creatine, there actually is an increased quantity in the muscles after short fasts.

With these considerations in mind we have deemed it important to study further the influence of high protein diets upon the creatine-creatinine metabolism of normal men and women.

Methods.

The plan of the experiments was to study the output of creatine, creatinine, and total nitrogen following the ingestion of diets alternately low and high in protein content, and free from creatine and creatinine. Most of the protein was ingested in the form of eggs, milk, and cheese, which while not containing as much arginine as certain of the vegetable proteins, such as were fed to pigs by McCollum and Steenbock,[6] nevertheless contain reasonable amounts of this amino-acid, and certainly enough to yield considerable creatine if the latter, under the conditions of the experiments, is a metabolic product of arginine. Basing our estimates upon analyses of Osborne and his coworkers[15] we have calculated that the ovalbumin and casein alone in our high protein diets, Nos. 2, 4, 6, and 10, contained 2 to 5 gm. of arginine per day.

[13] Mendel and Rose, *J. Biol. Chem.*, 1911–12, x, 255.

[14] Myers, V. C., and Fine, M. S., *J. Biol. Chem.*, 1913, xv, 283.

[15] Osborne, T. B., Jones, D. B., and Leavenworth, C. S., *Am. J. Physiol.*, 1909, xxiv, 252. Osborne, T. B., and Guest, H. H., *J. Biol. Chem.*, 1911, ix, 333.

The subjects for the experiments were healthy students in the School of Medicine of this University, and were engaged in practically the same amount of physical activity throughout the investigation. Total nitrogen was estimated by the Kjeldahl-Gunning method, and creatine and creatinine were determined by the micro methods of Folin.[16] Chemically pure creatinine zinc chloride served as the standard throughout. All picric acid used was previously purified according to the method of Folin and Doisy.[17]

TABLE I.

Composition of Diets.

No. of diet.	Articles of diets.							Food values.*	
	Bread.	Honey.	Butter.	Eggs.	Milk.	Cheese.	Apples.	N	Calories.
	gm.	gm.	gm.	gm.	cc.	gm.	gm.	gm.	
1	400	150	150					6.17	2,770
2	150			655	1,500	50		27.93	2,741
3	300	150	120					4.66	2,263
4	164			400	1,000	100		21.76	2,243
5	390	75	60					5.83	1,778
6	100			203	1,000	100		16.16	1,759
7	400		20	200		50		12.91	1,780
8	400	75	150	100	790		300	12.86	3,433
9	300	150	60				300	4.75	1,978
10	300		90	450	1,500	90	300	27.45	3,907

* The nitrogen and calorific values here tabulated were calculated from data given by Atwater, W. O., and Bryant, A. P., *U. S. Dept. Agric., Office Exp. Stations, Bull. 28* (revised), 1906.

EXPERIMENTAL AND DISCUSSION.

The composition, nitrogen content, and calorific value of each diet is indicated in Table I.

Tables II to V inclusive demonstrate the entire absence of effect of variations in the amount of protein in the diet upon the output of creatine and creatinine. Subjects in Tables II and III were women, and in Tables IV and V were men. Both series are entirely comparable with the exception that traces of creatine

[16] Folin, O., *J. Biol. Chem.*, 1914, xvii, 469.

[17] Folin, O., and Doisy, E. A., *J. Biol. Chem.*, 1916–17, xxviii, 349.

were eliminated irregularly by the women, while it was invariably absent from the urine of the men. In the women (Tables II and III) the output bore absolutely no relation to the quantity of

TABLE II.

Influence of High and Low Protein Diets on Creatine. Subject A. B., Female, Age 27 Years.

Date.	Body weight	Urine volume.	Reaction to litmus.	Total N.	Total creatinine.	Preformed creatinine.	Creatine as creatinine.	Remarks.
1917	*kg.*	*cc.*		*gm.*	*gm.*	*gm.*	*gm.*	
Oct. 16	54.8	2,150	Acid.	5.45	1.29	1.13	0.16*	Low N diet, No. 3. 4.66 gm. N and 2,263 calories.†
" 17		1,520	"	4.64	1.24	1.17	0.07	
" 18		1,865	"	4.90	1.21	1.15	0.06	
" 19		2,075	"	5.02	1.23	1.20	0.03	
" 20		2,005	"	4.47	1.16	1.12	0.04	
" 21		2,260	"	4.57	1.19	1.12	0.07	
Average		1,979		4.84	1.22	1.15	0.07	
Oct. 22	55.0	1,800	Acid.	11.83	1.26	1.22	0.04	High N diet, No. 4. 21.76 gm. N and 2,243 calories.
" 23		2,095	"	16.60	1.24	1.17	0.07	
" 24		1,610	"	17.03	1.17	1.17	0.00	
" 25		1,682	"	18.45	1.20	1.14	0.06	
" 26		1,927	"	19.62	1.29	1.22	0.07	
Average		1,823		16.71	1.23	1.18	0.05	
Oct. 27	54.6	1,972	Acid.	10.32	1.18	1.18	0.00	Low N diet, No. 3. 4.66 gm. N and 2,263 calories.
" 28		2,060	"	7.47	1.22	1.18	0.04	
" 29		1,663	"	6.18	1.15	1.15	0.00	
Average		1,898		7.99	1.18	1.17	0.01	

* Menstrual period ended Oct. 15.

† Began eating diet morning of Oct. 12.

protein in the diet. Similar irregularity in the excretion of creatine in women has recently been observed by Mary S. Rose,[18] who likewise was quite unable to note any relationship between the creatine output and the protein ingestion. The highest creatine excretion observed in our two experiments upon women was in Subject A. B. (Table II) on the 1st day of the experiment. The

[18] Rose, M. S., *J. Biol. Chem.*, 1917; xxxii, 1.

creatine on this day was 0.16 gm. expressed as creatinine, and the output of total nitrogen was 5.45 gm. As noted in the table, the menstrual period ended on the preceding day. This is interesting in view of the statement of Krause[19] that, "creatine is present in

TABLE III.

Influence of High and Low Protein Diets on Creatine. Subject E. B., Female, Age 28 Years.

Date.	Body weight.	Urine volume.	Reaction to litmus.	Total N.	Total creatinine.	Preformed creatinine.	Creatine as creatinine.	Remarks.
1917	kg.	cc.		gm.	gm.	gm.	gm.	
Oct. 17	59.1	1,290	Acid.	6.59	1.23	1.14	0.09	Low N diet, No. 5. 5.83 gm. N and 1,778 calories.†
" 18		1,270	"	6.57	1.20	1.12	0.08	
" 19		1,590	"	6.54	1.19	1.15	0.04	
" 20		1,650	"	5.78	1.11	1.06	0.05*	
" 21		1,025	"	5.93	1.11	1.07	0.04*	
Average....		1,365		6.28	1.17	1.11	0.06	
Oct. 22	58.2	1,335	Acid.	9.35	1.06	1.06	0.00*	High N diet, No. 6. 16.16 gm. N and 1,759 calories.
" 23		1,670	"	11.95	1.06	1.00	0.06	
" 24		1,315	"	13.47	1.03	0.98	0.05	
" 25		1,420	"	14.90	1.08	1.02	0.06	
" 26		1,040	"	14.80	1.09	1.05	0.04	
Average....		1,356		12.89	1.06	1.02	0.04	
Oct. 27	58.2	1,205	Acid.	9.78	1.02	0.99	0.03	Low N diet, No. 5. 5.83 gm. N and 1,778 calories.
" 28		1,045	"	7.00	1.08	1.08	0.00	
" 29		1,125	"	6.28	1.06	1.06	0.00	
Average....		1,125		7.68	1.05	1.04	0.01	

* Menstrual period.

† Began eating diet morning of Oct. 12.

the urine of women immediately after menstruation; it may or may not be present during the intermenstrual period." In Subject E. B. menstruation was apparently without effect upon the output of creatine, though the preformed creatinine was slightly greater during the few days preceding the onset of the

[19] Krause, R. A., *Quart. J. Exp. Physiol.*, 1911, iv, 293.

period. This difference, however, is entirely too small to permit one to draw any conclusions concerning it.

The fact that we are particularly interested in here is that contrary to the findings of Denis and Minot[9] *increases of 300 to*

TABLE IV.

Influence of High and Low Protein Diets on Creatine. Subject J. S. D. Male, Age 27 Years.

Date.	Body weight.	Urine volume.	Reaction to litmus.	Total N.	Total creatinine.	Preformed creatinine.	Creatine as creatinine.	Remarks.
1917	*kg.*	*cc.*		*gm.*	*gm.*	*gm.*	*gm.*	
Oct. 17	61.8	580	Acid.	5.61	1.64	1.64	0.00	Low N diet, No. 1. 6.17 gm. N and 2,770 calories.*
" 18		990	"	6.59	1.65	1.65	0.00	
" 19		750	"	7.06	1.58	1.58	0.00	
" 20		1,100	"	7.04	1.61	1.61	0.00	
" 21		1,180	"	6.76	1.64	1.64	0.00	
Average....		920		6.61	1.62	1.62	0.00	
Oct. 22	62.3	770	Acid.	9.28	1.64	1.64	0.00	High N diet, No. 2. 27.93 gm. N and 2,741 calories.
" 23		1,025	"	18.33	1.61	1.61	0.00	
" 24		1,550	"	21.68	1.55	1.55	0.00	
" 25		1,350	"	21.29	1.55	1.55	0.00	
" 26		1,430	"	23.17	1.62	1.62	0.00	
Average....		1,225		18.75	1.59	1.59	0.00	
Oct. 27	62.5	710	Acid.	14.36	1.62	1.62	0.00	Low N diet, No. 1. 6.17 gm. N and 2,770 calories.
" 28		580	"	11.08	1.67	1.67	0.00	
" 29		900	"	9.80	1.54	1.54	0.00	
Average....		730		11.75	1.61	1.61	0.00	

* Began eating diet morning of Oct. 12.

400 per cent in the protein intake are entirely without effect upon the excretion of creatine in women as well as in men. Nor do we believe that the failure of creatine to appear in the urine either in men or women can be explained by assuming that the muscles had not attained a condition of saturation. Surely it is reasonable to believe that the ingestion of such large amounts of protein as in Diets 2 and 4 would be adequate to saturate completely the muscles with creatine during the five day periods of the

experiments, if this substance were formed in the manner indicated by the Denis hypothesis.

To test the influence of the character of the diet upon the crea-

TABLE V.

Influence of High and Low Protein Diets on Creatine. Subject H. L. B., Male, Age 23 Years.

Date.	Body weight.	Urine volume.	Reaction to litmus.	Total N.	Total creatinine.	Preformed creatinine.	Creatine as creatinine.	Remarks.
1917	*kg.*	*cc.*		*gm.*	*gm.*	*gm.*	*gm.*	
Oct. 17	66.8	480	Acid.	6.47	1.67	1.67	0.00	Low N diet, No. 1.
" 18		570	"	7.18	1.73	1.73	0.00	6.17 gm. N and 2,770
" 19		720	"	7.92	1.58	1.58	0.00	calories.*
" 20		635	"	7.47	1.52	1.52	0.00	
" 21		520	"	6.79	1.54	1.54	0.00	
Average....		585		7.17	1.61	1.61	0.00	
Oct. 22	67.3	1,470	Acid.	15.91	1.67	1.67	0.00	High N diet, No. 2.
" 23		1,400	"	19.74	1.62	1.62	0.00	27.93 gm. N and 2,741
" 24		1,330	"	21.67	1.52	1.52	0.00	calories.
" 25		1,335	"	23.52	1.63	1.63	0.00	
" 26		1,325	"	23.81	1.64	1.64	0.00	
Average....		1,372		20.93	1.62	1.62	0.00	
Oct. 27	67.3	715	Acid.	13.19	1.56	1.56	0.00	Low N diet, No. 1.
" 28		510	"	10.16	1.58	1.58	0.00	6.17 gm. N and 2,770
" 29		490	"	8.90	1.55	1.55	0.00	calories.
Average....		572		10.75	1.56	1.56	0.00	

* Began eating diet morning of Oct. 12.

tine output further, two additional series of experiments were conducted. In one series the nitrogen intake was maintained constant throughout, while the calorific value of the diet was altered. In the other series a low protein-low calorific diet was followed by a high protein-high calorific diet. The results of one experiment only of each of these series are here presented. The data are tabulated in Tables VI and VII. It will be observed that *doubling the calorific intake, with the protein ingestion either*

unchanged or increased five-fold, invariably failed to induce the elimination of creatine. It is true that in both experiments there was a suggestion of an increase in the amount of preformed

TABLE VI.

Influence of High and Low Calorific Diets on Creatine. Subject H. L. B., Male, Age 23 Years.

Date.	Body weight.	Urine volume.	Reaction to litmus.	Total N.	Total creatinine.	Preformed creatinine.	Creatine as creatinine.	Remarks.
1917	kg.	cc.		gm.	gm.	gm.	gm.	
Dec. 7	67.3	925	Acid.	13.48	1.61	1.61	0.00	Low calorific diet, No.
" 8		830	"	13.23	1.58	1.58	0.00	7. 12.91 gm. N and
" 9		730	"	12.47	1.58	1.58	0.00	1,780 calories.†
" 10		730	"	13.65	1.58	1.58	0.00	
" 11		735	"	14.40	1.62	1.62	0.00	
" 12		735	"	13.82	1.55	1.55	0.00	
Average....		781		13.51	1.59	1.59	0.00	
Dec. 13	65.9	630	Acid.	11.57*	1.61	1.61	0.00	High calorific diet, No.
" 14		670	"	10.73*	1.67	1.67	0.00	8. 12.86 gm. N and
" 15		770	"	10.46*	1.67	1.67	0.00	3,433 calories.
" 16		830	"	9.91*	1.56	1.56	0.00	
Average....		725		10.67	1.63	1.63	0.00	
Dec. 17	66.4	680	Acid.	10.63	1.57	1.57	0.00	Low calorific diet, No.
" 18		700	"	11.42	1.61	1.61	0.00	7. 12.91 gm. N and
" 19		670	"	12.14	1.58	1.58	0.00	1,780 calories.
" 20		720	"	13.23	1.58	1.58	0.00	
Average....		692		11.86	1.59	1.59	0.00	

* The diminution in the nitrogen output during this period is evidently due to the sparing action of the increased carbohydrate intake.

† Began eating diet at noon Dec. 3.

creatinine, but this is so exceedingly small as scarcely to warrant making deductions concerning it. Indeed, both tables show days during the high calorific diets in which the creatinine output was less than on certain other days during the low calorific periods.

In the light of our experiments we are forced to the conclusion that *the theory which attributes the excretion of creatine on a creatine-*

free diet to an exogenous origin in the protein of the diet is untenable. This fact is true of the female as well as of the male subject. Ingestion of protein in amounts five or six times that necessary

TABLE VII.

Influence of a High Protein-High Calorific Diet and a Low Protein-Low Calorific Diet on Creatine. Subject J. S. D., Male, Age 27 Years.

Date.	Body weight.	Urine volume.	Reaction to litmus.	Total N.	Total creatinine.	Preformed creatinine.	Creatine as creatinine.	Remarks.
1918	*kg.*	*cc.*		*gm.*	*gm.*	*gm.*	*gm.*	
Jan. 12	63.0	605	Acid.	6.13	1.58	1.58	0.00	Low N-low calorific diet, No. 9, 4.75 gm. N and 1,978 calories.†
" 13		650	"	6.93	1.63	1.63	0.00	
" 14		350	"	6.26	1.60	1.60	0.00	
" 15		970	"	8.11*	1.58	1.58	0.00	
Average....		644		6.86	1.60	1.60	0.00	
Jan. 16	62.9	1,220	Acid.	14.36	1.68	1.68	0.00	High N-high calorific diet, No. 10, 27.45 gm. N and 3,907 calories.
" 17		1,370	"	17.72	1.61	1.61	0.00	
" 18		1,540	"	18.55	1.60	1.60	0.00	
" 19		1,370	"	19.80	1.61	1.61	0.00	
" 20		1,350	"	21.04	1.62	1.62	0.00	
Average....		1,370		18.29	1.62	1.62	0.00	
Jan. 21	63.1	950	Acid.	13.17	1.54	1.54	0.00	Low N-low calorific diet, No. 9, 4.75 gm. N and 1,978 calories.
" 22		940	"	8.27	1.51	1.51	0.00	
" 23		420	"	7.10	1.56	1.56	0.00	
" 24		870	"	7.77*	1.60	1.60	0.00	
Average....		795		9.08	1.55	1.55	0.00	

* The excretion of total nitrogen throughout the low nitrogen-low calorific periods indicates that the diet was not sufficient to meet the body's needs. Particularly is this manifested on Jan. 15 and 24 when the urinary nitrogen showed a tendency to increase in amount. Despite the change from a deficient diet to one far in excess of the anabolic requirements, creatine was absent throughout.

† Began eating diet morning of Jan 8.

to meet the nitrogenous needs of the organism entirely fails in the normal individual to induce the excretion of creatine, whether the high protein diet be excessive likewise in calories or not. The failure in the frequent attempts to find a precursor of creatine

by the *per os* or parenteral introduction of arginine, guanidine, methyl guanidine, and other closely related substances,[20] likewise points to the fact that the synthesis of creatine is either an anabolic reaction limited quantitatively to the needs of the body for creatine, or is a catabolic reaction associated with the disintegration of tissue protein alone. In either case its formation is quite independent of the protein of the diet. We have investigations now planned which we believe will throw more light upon the origin of creatine and its anhydride.

We are frank to admit that no theory at present adduced is adequate to explain the excretion of creatine in women and children. Certainly there seems to be little occasion for assuming a carbohydrate deficiency in these cases. We contend, however, that an explanation of this peculiar metabolic difference between the sexes on the one hand, and between children and adults on the other, must be sought in the endogenous metabolism rather than in excessive protein of the diet.

CONCLUSIONS.

1. The ingestion of diets excessively high in protein fails to induce the excretion of creatine in normal women and men.

2. Diets yielding 3,400 to 3,900 calories per day, whether accompanied by a moderate (13 gm.) or large (27 gm.) nitrogen intake, exert no appreciable influence upon creatine-creatinine metabolism.

3. It is our belief that no evidence has yet been adduced sufficient to justify the acceptance of a theory which postulates an exogenous origin of urinary creatine in the absence of creatine in the diet.

[20] Achelis, W., *Z. physiol. Chem.*, 1906, l, 10. Dorner, G., *ibid.*, 1907, lii, 225. Myers, V. C., and Fine, M. S., *J. Biol. Chem.*, 1915, xxi, 389. Baumann, L., and Marker, J., *ibid.*, 1915, xxii, 49.

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